From the Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

ROLE OF ANTIMICROBIAL PEPTIDES IN COMBATING SHIGELLOSIS AND IN ANTIBIOTIC-ASSOCIATED DIARRHEA

Protim Sarker

Karolinska Institutet

Stockholm 2012

Published by Karolinska Institutet.

ISBN 978-91-7457-751-8

Printed by
REPROPRINT AB
Stockholm 2012
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

To my family

ABSTRACT

Antimicrobial peptides (AMPs) constitute front-line components of innate immunity in multicellular organisms. AMPs are able to kill a wide range of pathogens and exhibit additional important functions such as chemotaxis, angiogenesis and wound healing. These peptides are constitutively expressed in immune and/or epithelial cells. However, their expression can also be induced or suppressed by different stimuli in a cell and tissue specific manner. In this thesis, the functional relevance of cathelicidins and defensins, two major families of AMPs in mammals, was studied in the context of enteric infectious diseases. Induction of these AMPs was explored in *Shigella* infection. The effect of antibiotics on the expression of AMPs in colonic epithelial cells was also investigated and the implication of this effect on *Clostridium difficile* associated diarrhea (CDAD) was assessed.

In a rabbit model of shigellosis, cathelicidin CAP-18 was downregulated in the large intestinal epithelia. Oral treatment with sodium butyrate (NaB) counteracted this downregulation and led to the conversion of the proform of CAP-18 into its active form in the stool (paper I). These findings correlated with reduced *Shigella* load in the intestinal lumen and clinical as well as histopathological recovery. Association between CAP-18 induction and reduction of bacterial load was supported by partial blocking of the antimicrobial activity in stool extract of a butyrate treated rabbit with CAP-18 specific antibody along with *in vitro* shigellacidal activity of CAP-18 peptide. In patients with shigellosis, administration of NaB enema as adjunct to antibiotic therapy led to an early improvement of rectal histopathology along with early reduction of inflammatory cells and proinflammatory cytokines in the stool compared to placebo treated patients (paper II). NaB treatment also resulted in enhanced expression of the human cathelicidin LL-37 in the rectal epithelia and sustained secretion of LL-37 in the stool. Since all patients were treated with antibiotics, attenuation of *Shigella* load in stool and clinical symptoms occurred simultaneously in both group of patients. Sodium-4-phenyl-butyrate (PB), an analogue of butyrate, was demonstrated to exhibit a similar therapeutic efficiency as NaB in the rabbit model of shigellosis (paper III). Downregulation of CAP-18 expression was also observed in the epithelia of lung and trachea. This suppression could render the respiratory tract susceptible to secondary infections during shigellosis. After oral treatment with PB or NaB, CAP-18 reappeared in the lung epithelia, which might strengthen the immunity of the respiratory tract against opportunistic respiratory pathogens.

Ciprofloxacin and clindamycin were found to suppress butyrate-induced expression of LL-37 in the colonic epithelial cell line, HT-29 (paper IV). *In vivo* inhibitory effect of ciprofloxacin was observed on CAP-18 expression in the rectal epithelia of *Shigella*-infected rabbits treated with NaB as well as of healthy rabbits. Induction of genes encoding HBD-3 and additional AMPs by NaB were also inhibited by ciprofloxacin. *In vitro* antibacterial activity of LL-37 against ciprofloxacin-resistant *C. difficile* indicates that the reduction of AMPs after antibiotic treatment may allow the overgrowth of *C. difficile* in the gut.

In conclusion, induction of AMPs is a promising therapeutic strategy against shigellosis. Suppression of AMP expression by antibiotics may contribute to CDAD, which is classically known to occur through disruption of the intestinal microbiota after antibiotic treatment.

LIST OF PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals

I. Raqib R, **Sarker P**, Bergman P, Ara G, Lindh M, Sack DA, Nasirul Islam KM, Gudmundsson GH, Andersson J, Agerberth B. Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. *Proc Natl Acad Sci U S A., 2006; 103(24):9178-83*

II. Raqib R, **Sarker P**, Mily A, Alam NH, Arifuzzaman ASM, Rekha RS, Andersson J, Gudmundsson GH, Cravioto A, Agerberth B. Efficacy of sodium butyrate adjunct therapy in shigellosis: A randomized, double-blind, placebo-controlled clinical trial. *Submitted to BMC Infectious Diseases*

III. **Sarker P**, Ahmed S, Tiash S, Rekha RS, Stromberg R, Andersson J, Bergman P, Gudmundsson GH, Agerberth B, Raqib R. Phenylbutyrate counteracts *Shigella* mediated downregulation of cathelicidin in rabbit lung and intestinal epithelia: A potential therapeutic strategy. *PLoS ONE, 2011; 6 (6): e20637*

IV. **Sarker P**, Al-Mamun A, Mily A, Jalal S, Bergman P, Gudmundsson GH, Raqib R, Agerberth B. Ciprofloxacin inhibits induction of antimicrobial peptide in colonic epithelial cells– implications for antibiotic associated diarrhoea caused by *Clostridium difficile. Manuscript*

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

I. **Sarker P**, Bhuiyan TR, Qadri F, Alam NH, Wretlind B, Bishop AE, Mathan M, Agerberth B, Andersson J, Raqib R. Differential expression of enteric neuroimmune-network in invasive and acute watery dirrhoea. *Neurogastroenterol Motil, 2010; 22 (1): 70-8, e29*

II. Sayem MA, Ahmad SM, Rekha RS, **Sarker P**, Agerberth B, Talukder KA, Raqib R. Differential host immune responses to epidemic and endemic strains of *Shigella dysenteriae* type I. *J Health Popul Nutr, 2011; 29 (5): 429-37*

III. Rahman MJ, **Sarker P**, Roy SK, Ahmad SM, Chisti J, Azim T, Mathan M, Sack D, Andersson J, Raqib R. Effects of zinc supplementation as adjunct therapy on the systemic immune responses in shigellosis. *Am J Clin Nutr 2005; 81(2): 495-502*

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid
AAD	Antibiotic-associated diarrhea
AD	Atopic dermatitis
AMP	Antimicrobial peptide
AP-1	Activator protein 1
5-ASA	5-aminosalicylic acid
ASE	Airway surface epithelia
ASF	Airway surface fluid
BM	Bone marrow
CAMP	Cathelicidin antimicrobial peptide
CAP-18	Cationic antimicrobial protein-18
CCR	CC chemokine receptor
CD	Crohn's disease
CDAD	*Clostridium difficile* associated diarrhea
CDI	*Clostridium difficile* infection
CF	Cystic Fibrosis
CFTR	CF transmembrane conductase regulator
CFU	Colony forming unit
CHX	Cycloheximide
COX-2	Cyclooxygenase-2
CPS	Capsular polysaccharide
CRAMP	Cathelicidin related antimicrobial peptide
DAI	Disease activity index
DC	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FC	Fulminant colitis
FPRL	Formyl peptide receptor like
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GM-CSF	Granulocyte colony stimulating factor
GPCR	G-coupled protein receptor
HBD	Human β-defensin
hCAP	Human cationic antimicrobial protein
HD	Human defensin
HDACi	Histone deacetylase inhibitor
HIF-1α	Hypoxia-inducible factor 1, α subunit
HNP	Human neutrophil peptide
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
iEC	Intestinal epithelial cell
IFN-γ	Gamma interferon
IGF	Insulin-like growth factor
IL	Interleukin

Ipa	Invasion plasmid-coded antigen
KO	Knock-out
LCA	Lithocolic acid
LOS	Lipooligosaccharide
LP	Lamina propria
LPS	Lipopolysaccharide
M cell	Microfold cell
MAPK	Mitogen-activated protein kinase
MBD	Mouse β-defensin
mCRAMP	Mouse CRAMP
MPO	Myeloperoxidase
NaB	Sodium butyrate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κB
NK cell	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerisation domain
O-SP	O-specific polysaccharide
PAI	Pathogenicity island
PAMP	Pathogen-associated molecular pattern
PB	Sodium-4-phenylbutyrate
PBMC	Peripheral blood mononuclear cell
PBP1a	Penicillin-binding protein 1a
PMC	Pseudomembranous colitis
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
RBC	Red blood cell
rCRAMP	Rat CRAMP
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SE	Surface epithelia
SGD	Specific granule deficiency
STAT	Signal Transducer and Activator of Transcription
TGF-α	Transforming growth factor-α
Th cell	T-helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TSA	Trichostatin A
TTSA	Type III secretion apparatus
UC	Ulcerative colitis
VDR	Vitamin D receptor
VDRE	Vitamin D response element

1 BACKGROUND

1.1 SHIGELLOSIS

Shigellosis, or bacillary dysentery, is caused by the infection of the large intestinal tract by bacteria of the genus *Shigella*. *Shigella* is principally a human pathogen, which is transmitted via fecal/oral route. The bacteria enter into the body through the ingestion of contaminated water or food. The disease is very infectious as 10-100 bacteria are sufficient to cause infection. After 12-96 hours of bacterial encounter, the disease symptoms are manifested, which range from mild watery diarrhea to severe inflammatory colitis. Frequent passage of scanty loose stools containing mucus and/or blood is the classical manifestation of shigellosis. Patients with severe shigellosis experience fever, abdominal cramp and tenesmus. Mild shigellosis is self-limiting and typically lasts for 4-7 days but appropriate medication is required for severe cases. Some infected persons may remain as asymptomatic carrier.

Shigellosis is regarded as one of the major public health burden, especially in resource-poor countries. Although, case fatality rate has decreased significantly over the last decade, the incidence rate of endemic shigellosis still remains very high, which is estimated to be about 125 million cases every year in Asia [1]. Children below 5 years of age are more susceptible to fatal shigellosis. The childhood death in shigellosis due to colitis is often aided by malnutrition, septicemia and secondary respiratory complications such as pneumonia [2-4]. Malnutrition and persistent diarrhea are two major long-term consequences of shigellosis. Loss of serum proteins during shigellosis [5], results in linear growth stunting in young children who do not have access to adequate dietary protein [6]. Moreover, infection with *Shigella* is a risk factor for post-infectious complications, such as reactive arthritis [7] and post-infectious irritable bowel syndrome (PI-IBS) [8].

Shigella is a member of the tribe *Escherichia* in the family *Enterobacteriaceae*. It is a gram negative, uncapsulated, nonsporulating, nonmotile, facultative anaerobic bacillus. *Shigella* is divided into serogroups and subdivided into serotypes based on antigenic difference in the O-specific polysaccharide (O-SP) of lipopolysaccharide (LPS) present in the outer membrane. There are 4 species of *Shigella*: *S. dysenteriae* (group A, 17 serotypes), *S. flexneri* (group B, 14 serotypes), *S. boydii* (group C, 20 serotypes) and *S. sonnei* (1 serotype). Three strains are mainly responsible for the occurrence of shigellosis: *S. dysenteriae* type 1, *S. flexneri* 2a and *S. sonnei.* The first two strains are encountered in developing countries and generally cause severe dysentery. *S. sonnei* is found to cause mild diarrheal illness, mostly in industrialized countries. *S. dysenteriae* type 1 is the only strain found in epidemic and also pandemic shigellosis.

1.1.1 Pathogenesis

Shigella pathogenesis is a multistep process, involving invasion and colonization of large intestinal epithelial cells (iEC) and subsequent destruction of the mucosa through excessive inflammation (Figure 1). *Shigellae* are very inefficient in invading iEC through the apical route [9]. Instead, the bacteria enter into the microfold (M) cells of the Peyer's patches and are translocated to the underlying subepithelial pocket [10, 11].

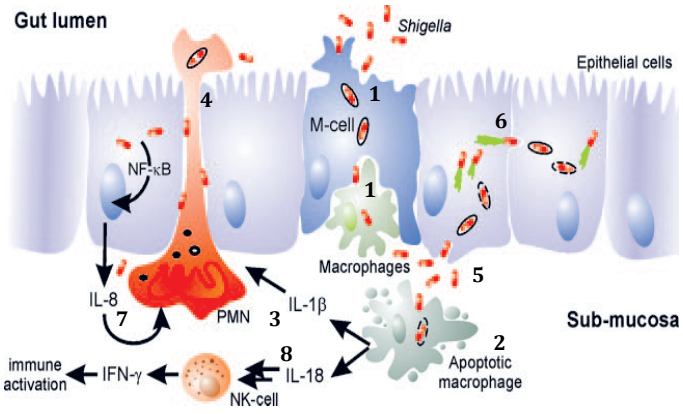


Figure 1: Schematic representation of *Shigella* pathogenesis. (1) *Shigellae* cross the intestinal epithelial cell (iEC) barrier by transcytosis through M cells and encounter resident macrophages. (2) Bacteria are released in the submucosa by inducing death of macrophages. (3) IL-1β is released from the dying macrophages, recruiting PMNs to the infection site. (4) Recruited PMNs disrupt iEC tight junctions, facilitating paracellular entry of the bacteria. (5) *Shigellae* invade iEC from basolateral site. (6) Bacteria spread to adjacent cells. (7) Infected iEC produce IL-8, forming a positive feedback loop to exacerbate inflammation. (8) IL-18, released from dying macrophages, stimulates NK cells to produce IFN-γ, leading to protective immune responses. Adopted from reference [12] with minor modifications.

Within the pocket, *Shigellae* are engulfed by resident macrophages, multiply in cytoplasm and disseminate into submucosa by inducing cell death [10, 11, 13, 14]. The bacteria also induce release of mature interleukin-1β (IL-1β) and interleukin-18 (IL-18) from the dying macrophages, triggering an early inflammation [15-17]. The resulting infiltration of polymorphonuclear neutrophils (PMNs) to the infection site breaks the epithelial barrier integrity and thus allows more luminal bacteria to accumulate in the submucosa without the help of M cells [10]. *Shigellae* then invade iEC from the basolateral site by inducing macropinocytosis and escape into the cytoplasm by rapturing vacuole membrane and replicate therein [9, 18]. Utilizing actin-based motility, the bacteria move intracellularly and pass to adjacent iEC, leading to their spread throughout the tissue [18]. Large array of proinflammatory cytokines and chemokines including IL-8 is induced and secreted from the infected iEC, which disseminate the inflammation [18-20]. The spread of inflammation is characterized by massive influx of PMNs, additional immune cells and accumulation of proinflammatory cytokines and other innate mediators [21-26]. *Shigella* infection eventually leads to death of host cells by apoptosis and/or necrosis [22, 27]. The consequence of the overall events is huge mucosal damage as evident by epithelial erosion, mucosal abscesses and hemorrhages in acute shigellosis [10, 22, 28]. Inflammatory response may also persist for a long time after the recovery from clinical symptoms, giving rise to chronic illness [23, 25].

1.1.2 Immune responses and immune evasion

Shigellae encounter several host defense mechanisms on the way to cause infection. Virulent *Shigellae* are highly adaptable to overcome these defenses. The bacteria are highly tolerant to the acidic environment [29], hence easily pass through the stomach into the intestine. As mentioned in the previous section, *Shigellae* breach the physical barrier of intestinal epithelial lining by exploiting M cells and resident macrophages in the subepithelial dome of the lymphoid follicles. The bacteria can also overcome the chemical barrier of the epithelia by inhibiting the production of effector molecules such as antimicrobial peptides or proteins [30, 31]. By colonizing iEC, *Shigellae* avoid extracellular components of the host immune system such as antibodies or complement factors. Self-renewal of intestinal epithelium in every 4-5 days [13] provides a functional barrier against bacterial colonization through shedding of infected cells. *Shigellae* can control this rapid turnover of epithelial cells by reducing the cell cycle progression of stem cells and by hindering exfoliation of infected iEC [13]. Cytosolic pathogens encounter another host defense mechanism called autophagy. Autophagosome degrades own components of the cell via lysosomal fusion as part of cellular homeostasis, but can also target and destroy intracellular pathogens and/or infected cells [32]. *Shigella* invasion triggers autophagy but the bacteria evade autophagic recognition [33]. Although, migrated PMNs initially facilitate translocation of *Shigellae* to the submucosa, the bacteria are eventually killed by the PMNs [21]. In addition to intracellular killing, PMNs form neutron extracellular traps (NETs). NETs are composed of chromatin fibers, filled with antimicrobial peptides and other granule proteins that entrap extracellular *Shigellae* [34]. This leads to degradation of bacterial virulence-associated proteins and subsequent killing of the bacteria. Besides NETs, secreted innate immune effectors and inflammatory mediators from immune cells may also contribute to *Shigellae* clearance [24]. However, *Shigellae* execute various strategies to reduce excessive inflammation for their survival. The bacteria induce death of the immune cells [22, 27], counterbalance pro-inflammatory responses [35] as well as induce the production of antinflammatory mediators [23]. Gamma interferon (IFN-γ) is a critical cytokine for immunity against viral and intracellular bacterial pathogens [36]. Natural killer (NK) cell-derived IFN-γ was shown to resist pulmonary *Shigella* infection in a mice model through induction of intracellular killing by macrophages [37]. Interestingly, IL-18, released from the dying macrophages during early *Shigella* infection promotes IFN-γ production from NK cells (Figure 1) [12]. Upregulation of IFN-γ production and enhanced expression of IFN-γ receptors during the recovery phase of shigellosis suggests the importance of IFN-γ in controlling *Shigella* infection in humans [38]. However, decreased expression of IFN-γ receptors, reduced secretion and extracellular deposition as well as systemic downregulation of IFN-γ at the acute stage of shigellosis indicate another immune escape strategy of *Shigella* during early stage of the infection [38-40].

Following infection, both systemic and mucosal humoral immune responses are augmented against *Shigella* antigens such as lipopolysaccharide (LPS) and invasion plasmid-coded antigens (Ipa) [41, 42]. Mucosal IgA antibodies to Ipa are associated with protection from the disease [43]. Induced mucosal and serum antibodies, specific for LPS protect re-infection by the same *Shigella* serotype [44, 45]. We have demonstrated that humoral responses in infected children are reduced and delayed compared to adults in an endemic setting [42]. In healthy endemic population, the

levels of serum antibodies to LPS and Ipa increase with age [42, 46]. Moreover, serum antibody levels to both antigens in naïve population from non-endemic areas are significantly low compared to that with endemic population [42, 46]. These findings indicate that several episodes of infection are needed for building humoral protective immunity and explain why children are more susceptible to *Shigella* infection. Cell mediated adaptive immune responses in shigellosis have not been well characterized so far. T cells are infiltrated and activated in the intestinal mucosa during shigellosis [26, 47], but their responses to *Shigella* remains poorly understood. It has recently been demonstrated that antigen-specific $CD8^+$ T cells are not primed during *Shigella* infection [48]. $CD4^+$ T cells are invaded by *Shigellae* and do not respond to chemokine-induced migration [49]. However, in a murine pulmonary infection model, *Shigella*-primed Th17 cells was shown to exhibit protective immune response against the same strain up to 12 months after priming [50].

1.1.3 Virulent factors responsible for pathogenesis and immune evasion

Secretion of several diverse effectors via type III secretion apparatus (TTSA) is the principle virulent determinants of *Shigella.* The majority of the effectors and TTSA are encoded by a large (~ 200 kb) virulence plasmid [12]. A conserved 31 kb entry region of the plasmid is considered as the main *Shigella* pathogenicity island (PAI). This region encodes (i) the components of the TTSA (mxi-spa locus), (ii) substrates of TTSA (the translocators IpaB and IpaC and the effectors IpaD, IpgB1, IpgD and IcsB), (iii) chaperons (IpgA, IpgC, IpgE and Spa15) to which the substrates remain bound inside bacterial cytoplasm before being secreted and (iv) transcriptional activators (VirB and MxiE) [51]. Other substrates of TTSA, i.e. VirA, OspB-G, and IpaH are encoded outside the PAI region. Five other virulent proteins encoded by plasmid are IcsA (VirG), IcsP, VirK, MsbB2 and SepA [51]. Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) of the host is also crucial for the pathogenesis and immune responses elicited by *Shigella* [13, 35].

IpB mediates apoptosis of macrophages by activating caspase-1[52]. Sensing of bacterial components by nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) also leads to pyroptosis of macrophages [35]. Activated caspase-1 hydrolyzes pro-IL-1β and pro-IL-18 and thus allows the release of mature IL-1β and IL-18 [15]. Delivery of a subset of effectors including IpaA, IpaB, IpaC, IpgB1, IpgB2, IpgD and VirA promotes basolateral entry of bacteria into the iEC cells through formation of membrane-ruffles [53]. VirG and VirA mediate intra- and inter-cellular movement of the bacteria through actin polymerization and degradation of microtubules [53]. IcsB, by binding to VirG prevent recognition of VirG by autophagy related protein 5 (Atg5) and hence evade autophagy [33]. The binding of peptidoglycan fragments of the bacteria to NLRs triggers the production of proinflammatory cytokines through the activation of nuclear factor κB (NF- κB) [35]. IpaH, OspG, OspF and OspZ downregulate host inflammatory responses by inhibiting NF-κB activation [35]. The secretion of IpaB slows down the cell cycle progression of epithelial progenitor cells [13]. iEC exfoliation/ detachment is inhibited by OspE [13]. IpgD impairs the chemokine-induced migration of $CD4^+$ T cells [49].

1.1.4 Treatment

WHO recommends that all episodes of bloody diarrhea should be treated with antibiotics and the choice of antibiotics should be based on local or at least on regional susceptibility data [54]. Nalidixic acid, ampicillin, chloramphenicol, co-trimoxazole and tetracycline, which were used in the past, are no longer recommended, because of widespread resistance of *Shigella* spp. to these antibiotics. In the absence of local or regional data, WHO recommends ciprofloxacin as the first choice of antibiotic for patients with bloody diarrhea, irrespective of their age. Fluoroquinolones other than ciprofloxacin, pivmecillinam and ceftriaxone are recommended as second line antimicrobials for all age group, while azithromycin is recommended only for adults. However, resistant *Shigella* strains to ceftriaxone and fluoroquinolones including ciprofloxacin have been emerging in recent years [55, 56]. The usage of rehydration therapy for all age groups and zinc supplementation for children up to 5 years of age are recommended for diarrheal diseases including shigellosis.

1.1.5 Preventive measures and vaccines

Provision of clean water, proper nutrition and good hygiene practice are the critical preventive measures against shigellosis as well as other infectious diarrheal diseases.

For several decades, attempts have been made to develop *Shigella* vaccine but a safe and protective vaccine is still elusive. *Shigella* vaccine candidates are broadly categorized as killed whole bacterium, live attenuated and subunit vaccines. A number of live attenuated vaccine candidates have been developed against *S. flexneri* [57]. Among these candidates, SC602, at 10^4 cfu dose, was shown to be safe and to induce strong immune responses in US volunteers [58]. It also conferred protection to a subgroup of volunteers, who were challenged with parental strain two months later [58]. However, a recent double-blind, randomized clinical trial in an endemic setting in Bangladesh, has failed to show the immunogenicity of SC602 in adults and school children [59]. CVD 1207 and CVD 1208 also have shown promise in phase I clinical trials in USA [57]. SC599, a *S. dysenteriae* 1 vaccine candidate, have undergone successful phase I and II trials in European populations [60, 61]. A *S. sonnei* vaccine candidate, WRSS1 has been shown to be safe and immunogenic in US and Israeli volunteers [57].

Approaches for subunit vaccine are based on the use of protective antigen(s) such as LPS and Ipa proteins. *S sonnei* and/or *S flexneri* conjugate vaccines based on O-SP have been demonstrated to be safe and to stimulate LPS specific antibodies in adults, 4-7 years old children and in 1-4 years old children [62]. Vaccine based on *Shigella* invasion complex (Invaplex, a complex of LPS, IpaB, IpaC and IpaD), have been shown to exert minimum side effects and give rise to protective immune response in phase I clinical trials [62].

1.2 ANTIBIOTIC-ASSOCIATED DIARRHEA (AAD)

Antibiotics are the mainstay of therapy against bacterial infections. Besides, killing or inhibiting bacterial pathogens, antibiotics may upset the normal gut flora, allowing

opportunistic pathogens such as *Clostridium difficile, Clostridium perfringens, Staphylococcus aureus and Klebsiella oxytoca* to colonize the gut mucosa [63]. Infection by these pathogens may lead to mucosal deterioration and diarrhea. Disruption of the normal flora may also account for reduced microbial metabolism of carbohydrates and primary bile acids, leading to osmotic or secretory diarrhea. Moreover, antibiotics may directly alter the motility of the gut, causing diarrhea [63]. These diarrheal events due to antibiotic usage are collectively termed antibiotic-associated diarrhea (AAD). AAD constitutes 5-25% of all diarrheal incidences [63].

1.2.1 *Clostridium difficile* associated diarrhea (CDAD)

CDAD is on the top of the list among all AAD, accounting for 15-25% of all cases [64]. Clindamycin, ampicillin, amoxicillin, cephalosporin and fluroquinolones are the major antibiotics implicated in CDAD. Vancomycin and metronidazole, commonly used to treat CDAD, can also be responsible for subsequent CDAD. Long duration of antibiotic treatment and use of multiple antibiotics increase the risk of *Clostridium difficile* infection (CDI) [64]. Impairment of immune functions due to age, malnutrition, shock, HIV infection, severe underlying diseases, and usage of immunosuppressive and chemotherapeutic drugs is also a major predisposing factor for CDAD. Some chemotherapeutic drugs also affect the normal flora [64]. Inhibition of gastric acid secretion by the use of proton pump inhibitors (PPI) or H2 blockers contribute to CDI, probably due to increased survival of spores at elevated pH [64]. Inflammatory bowel disease (IBD) and bowel surgery are other important risk factors for CDAD [64].

Patients staying in health care facility for prolonged period are in risk of acquiring CDI, where pathogens can easily be transmitted and patients are often treated with antibiotics. Elderly people (>65 years) are more susceptible to CDAD because of impaired immunity, receiving antibiotics more often and possibility of being hospitalized for longer periods [64].

1.2.1.1 Transmission and pathogenesis

C. difficile is a gram-positive, obligate anaerobic, spore-forming bacillus. Spores can survive in the harsh environment for longer period and can withstand common sterilization methods including high temperature, ultraviolet light and chemicals. Spores are transmitted via the fecal-oral route, through the hands of patients, health care workers or visitors. In addition, contaminated commodes, bathtubs and medical equipment are the potential transmitters of the spores.

Ingested *C. difficile* spores germinate in the large intestine and vegetative forms multiply. Vegetative bacteria penetrate the mucus layer with the help of flagella and proteases and adhere to the iEC through multiple adhesins [65]. Adherent bacteria then produce toxins, which are the main virulent determinant of *C. difficile.* Virulent strains generally produce two exotoxins i.e. toxin A (TcdA) and toxin B (TcdB). Both toxins are cytotoxic, disrupting the actin cytoskeleton by uridine diphosphate/glucose dependent glycosylation of Rho GTPase family. [66]. This cytotoxic effect loosens the iEC tight junctions, leading to increased epithelial permiability and fluid accumulation. Toxins also induce iEC, mast cells and macrophages to release inflammatory mediators

and activate sensory nerves to release neuropeptides [66, 67]. These mediators chemoattract PMNs and other inflammatory cells and thereby induce strong inflammatory responses and exacerbate fluid accumulation [66]. Although both toxins cause disease, toxin B is more potent than toxin A in damaging epithelial barrier [68] and is essential for full expression of CDI in hamster model of CDAD [69]. Consistently, TcdA-TcdB+ strains were identified from patients with severe CDAD [70]. Some strains also produce a binary toxin (CDT), consisting of both binding and enzymatic components [71]. Through ADP ribosyl transferase activity, binary toxin can disorganize cytoskeleton and hence may enhance the severity of CDAD [71].

The CDI cycle including transmission, pathogenesis and risk factors is depicted in figure 2.

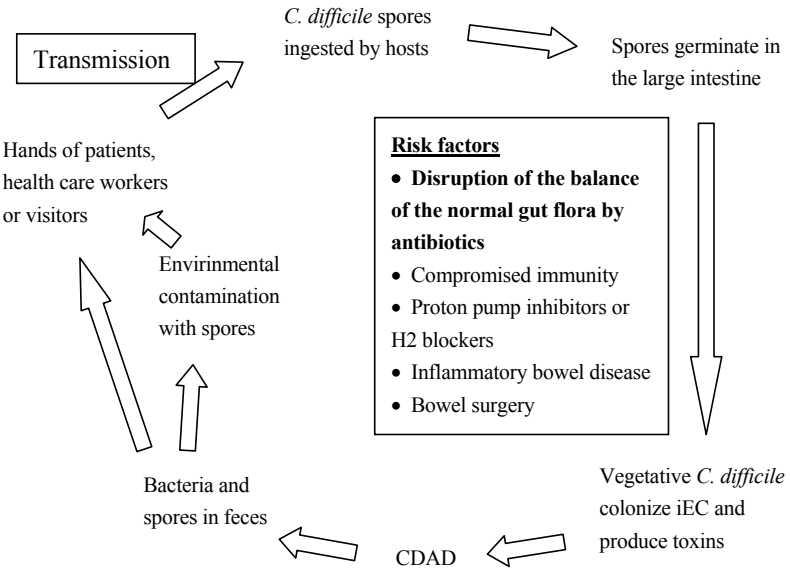


Figure 2: *Clostridium difficile* infection cycle. iEC: intestinal epithelial cells; CDAD: *C. difficile* associated diarrhea

1.2.1.2 Clinical spectrum

The clinical spectrum of *C. difficile* infection, in ascending order of severity, includes asymptomatic carriage, diarrhea, colitis without pseudomembrane formation (nonspecific colitis), pseudomembranous colitis (PMC) and fulminant colitis (FC) [72]. Diarrhea is sometimes accompanied by lower abdominal cramps and is generally resolved after stopping antibiotic treatment. Nonspecific colitis is the most common clinical feature of CDAD, characterized by malaise, abdominal pain, nausea, anorexia and watery diarrhea. PMC is manifested by abdominal cramps and profuse watery, green, foul smelling or bloody diarrhea with increased leukocytosis and enhanced number of fecal leukocytes. Multiple yellow-white friable plaques attached to underlying mucosa are observed endoscopically. Fibrinous exudate containing

lymphocytes, epithelial cells and mucin is the histopathological feature of PMC. FC is the most severe form of colitis often demonstrated by fever, severe lower quadrant or diffuse abdominal pain accompanied by distention, electrolyte disturbances, lactic acidosis, marked leukocytosis and hypoalbuminemia. FC often leads to bowel perforation, toxic megacolon and subsequent death.

Recurrent CDAD, reported in ~20% of patients, is another major complication of *C. difficile* infection [73]. Within a few days or weeks of stopping *C. difficile* treatment, symptoms of CDAD may re-appear. Repeated occurrence might be a result of persistence of imbalanced fecal flora due to repeated antibiotic treatment or of impaired immune responses.

1.2.1.3 Epidemiology

In recent years the incidence and severity of CDAD have dramatically increased [74]. Moreover, the infection is spreading to population previously considered at low risk including infants and children, healthy persons living in community and peripartum women [74]. The changing epidemiology of CDAD is largely due to the emergence of a new hypervirulent strain, accounting for several outbreaks in North America and Europe from the beginning of this century [65, 74]. This strain is designated as the North American pulse-field gel electrophoresis type 1 (NAP1), restriction endonuclease type BI, polymerase chain reaction (PCR) ribotype 027 (NAP1/BI/027). Epidemic hypervirulent 027 strain is resistant to fluoroquinolones, erythromycin and clindanycin. This toxinotype III strain produces high amount of toxin A and B and also contains binary toxin.

1.2.1.4 Preventive measures

According to the most recent (2010) clinical practice guidelines for CDI in adults by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), following preventive measures should be taken [75]. (1) To prevent the transmission of the pathogen, each patient with CDAD should be accommodated in a private room or at least be provided with a dedicated commode. Health care workers and visitors should use gloves and gowns when entering the room of a patient and must wash their hands with soap and water afterwards. Environmental decontamination should be routinely carried out with chlorine-containing or other sporicidal agents. (2) To minimize the risk of CDI, the frequency and duration of antibiotic therapy and number of antibiotics should be minimized.

1.2.1.5 Treatment

First step in treating patients with CDAD is to withdraw antibiotics being given for other purposes or at least changing antibiotic regimens. This strategy very often provides early resolution of diarrheal symptoms and even some cases of established PMC [64]. If this strategy does not work, patients should be treated with specific antibiotic therapy. According to the Clinical practice guidelines for CDI in adults by SHEA and IDSA, oral metronidazole is the drug of choice for initial mild or moderate episodes and for first recurrence [75]. This drug is not recommended for longer therapy

because of neurotoxicity. For initial severe episode or for second recurrence, oral vancomycin is recommended. Severe, complicated CDI is recommended to be treated with oral vancomycin with or without intravenous metronidazole [75]. Fidaxomicin, a macrolid antibiotic has been approved by FDA very recently for treatment of CDAD in adults [74]. Alternative approaches such as use of probiotics and immune therapy have been and is currently being evaluated. However, the usage of probiotics is not recommended by the SHEA and IDSA guidelines because of insufficient data and the risk of bloodstream infection [75]. In a phase II randomized clinical trial, co-administration of antibodies to toxin A and toxin B as adjunctive therapy to classical antibiotics was shown to decrease the recurrence rate of CDI [76]. Subtotal colectomy is recommended for severely sick patients, when lactate level rises to 5mmol/L and blood leukocyte counts increases to 50000cells/μL[75].

1.3 ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs), as the name implies, are peptides having antimicrobial activity. These peptides are able to kill a wide range of microorganisms including bacteria, viruses and fungi. AMPs are present in almost all living organisms including vertebrates, invertebrates, plants, insects and even in microorganisms, indicating that these peptides are evolutionary conserved [77]. In multicellular organisms, AMPs are important effector molecules of innate immunity. In 1981, Hans G Boman and coworkers discovered the first AMPs from the silk moth, *Hyalophora cecropia* [78]. They isolated and characterized two AMPs from the hemolymph of the moth and showed efficient killing capacity of these peptides against bacteria. They named the peptides cecropins based on the origin of the species. Within a few years, the mammalian AMPs were discovered in rabbit macrophages and human PMNs [79, 80]. Isolation and characterization of magainins in the skin of African clawed frog *Xenopus laevis* by Michael Zasloff is another revolutionary discovery in this research field [81]. The research field on AMPs then flourished and many AMPs from different sources have been and are currently being characterized. At present, more than 1200 AMPs have been characterized or predicted from gene sequences [82]. Several mimics of AMPs have also been developed [83].

AMPs are highly variable in their primary, secondary and tertiary structure, i.e. α-helical, β- sheet, extended helices rich in certain amino acids and loop structures [77]. However, there are some common features in their structures: these peptides are <100 amino acid (aa) residues long, cationic and amphipathic (one hydrophobic side and one ionic side). Positively charged AMPs bind to negatively charged components of microbial membrane such as phospholipid, LPS, lipoteichoic acid and peptidoglycan via electrostatic interaction [84]. The amphipathic nature of the peptides allows them to be incorporated into lipid bilayer of the membrane. This interaction destroys the membrane integrity and subsequently the microbes are lysed. Several mechanistic models have been developed to delineate the bacterial membrane disruption by AMPs [84]. In barrel-stave pore model and in toroidal pore model AMPs (generally α-helical peptides) form transmembrane pores in the bacterial membrane. In the carpet model, peptides accumulate on the bacterial surface and form a carpet-like structure that lie in parallel with the membrane. AMPs then disrupt the membrane in a detergent-like manner. In addition, some peptides target different intracellular components or

processes to kill microorganisms [84]. After being translocated into cytoplasm these peptides affect the cytoplasmic membrane septum formation, inhibit synthesis of cell wall/nucleic acids/proteins or hinder the enzymatic activities of the microbes. AMPs can also be cytotoxic to eukaryotic cells, but at much higher concentration than the bactericidal concentration [85]. The presence of neutral zwitterions and cholesterol in eukaryotic membrane results in a week interaction with AMPs [77].

Besides antimicrobial activity, several AMPs participate in immunomodulatory activities such as chemotaxis, activation and alteration of cytokine/chemokine responses of both innate and adaptive immune cells [86, 87]. AMPs thus form a bridge between innate and adaptive immune system. Additional functions attributed to these peptides are neutralization of LPS [88, 89], regulation of the normal flora [90], wound repair and maintenance of epithelial barrier integrity [91-95]. Because, of their multifaceted activities in host defense, AMPs are often termed as host defense peptides (HDPs) and for their immunomodulatory properties, they are also designated as alarmins.

1.3.1 Cathelicidin

Cathelicidin is one of the main families of AMPs in mammals [87], found also in fish [96], birds [97] and amphibians [98]. This diverse group of AMPs is gene-encoded and produced as preproprotein (128-143 amino acid residues), consisting of a conserved cathelin domain (99-114 residues), connected to an N-terminal signal peptide (29-30 residues) and a highly variable C-terminal domain (12-100 residues) [99]. Upon release of the signal peptide, the proprotein (precursor protein) is stored inside the cells. Based on the conservation of the cathelin domain, Zanetti *et al.* coined the term cathelicidins for this group of precursor proteins [99]. When the proprotein is released from activated cells, the C-terminal domain is cleaved off from the cathelin domain to generate active mature peptide [87]. The free cathelin protein inhibits cysteine protease activity and can also elicit antimicrobial activity [100]. Active cathelicidin peptides vary considerably in structure. They can be α-helical (such as LL-37, rabbit CAP-18), can form β-hairpins (such as pig protegrin 1-5) or can have extended helices due to abundance of certain amino acid residues (such as porcine PR-39) [87].

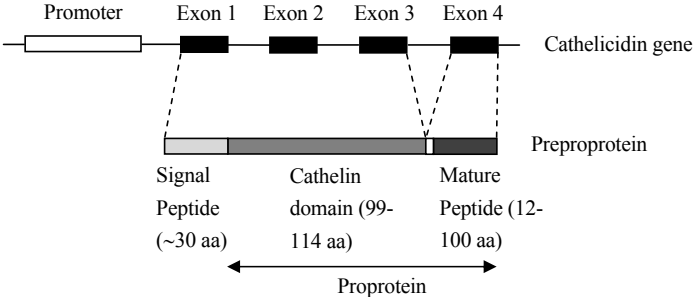


Figure 3: Schematic representation of cathelicidin genes and proteins. The genes consist of 4 exons. The first three exons encode the signal peptide and cathelin, while the fourth exon encodes the mature active peptide and the processing site.

The genes encoding cathelicidins consist of four exons. The first three exons encode the signal peptide and the cathelin domain and the fourth exon encodes the processing site and the antimicrobial peptide domain (Figure 3). The number of genes varies from species to species. For example, human, rabbit, mouse and rat have single gene, while cows, pigs and sheep have several genes [87].

1.3.1.1 LL-37 and its orthologues

LL-37 is the sole cathelicidin peptide in human. This peptide is 37 residues long and contains two leucine residues at the N-terminus and therefore named LL-37. The first clones encoding the peptide were obtained through screening of a human bone marrow cDNA library and the putative peptide was designated FALL-39 [101]. The FALL-39 transcript was detected in testis along with bone marrow by Northern blot analysis. In the following year, the mature peptide was isolated from degranulated PMNs and structural analysis revealed the peptide to be LL-37 [102]. Since then, LL-37 has been detected in additional cell types including monocytes, different lymphocytes, mast cells, eosinophils, dendritic cells, Langerhans cells and many epithelial cells [103-112]. The mammary, salivary and sweat glands have also been shown to express LL-37 [113-115]. The gene encoding proprotein of LL-37 is designated *CAMP* (cathelicidin antimicrobial peptide) and is located on chromosome 3p21.3 [116]. The proform is a 18 kDa protein and termed human cationic antimicrobial protein (hCAP)-18 [88]. The extracellular cleavage of hCAP-18, generating LL-37 is mediated by proteinase 3 in PMNs [117] and kallikrein 5 in skin epithelia [118]. Further processing of LL-37 by kallikrein 5 and 7 generates shorter peptides, exhibiting differential antimicrobial activity. In seminal plasma, the processing of hCAP-18 by gastricsin results in one residue longer peptide (ALL-38) [119].

The orthologues of LL-37 in rabbit is CAP-18 [89], in mouse is mCRAMP [120] and in rat is rCRAMP [121]. LL-37 and all its orthologues are α-helical in structure.

The expression of LL-37 or its orthologues can be constitutive or induced by different stimuli. Induced expression of LL-37 is observed in psoriatic lesions [107, 122] as well as in inflamed and non-inflammed colonic mucosa of patients with ulcerative colitis [123]. Induction of LL-37 has also been noted in bacterial and parasitic infections such as by *Staphylococcus aureus* in keratinocytes [124], *Helicobacter pylori* in gastric epithelial cells [111], uropathogenic *Escherichia coli* in epithelial cells of the urinary tract [112] and *Entamoeba histolytica* in colonic epithelial cells [125]. mCRAMP is upregulated by Group A *Streptococcus* (GAS) in keratinocytes [106], by uropthogenic *E. coli* in the tubular epithelial cells of kidney [112] by *Neisseria meningitidis* in blood brain barrier [126] and by *E. histolytica* in cecal epithelia [125]. The Growth factors, insulin-like growth factor-1 (IGF-1) and transforming growth factor-α (TGF-α) also induce the expression of LL-37 in keratinocytes [127]. Hypoxia-inducible factor 1, α subunit (HIF-1α) in myeloid cells [128] and reactive oxygen species (ROS) in macrophages [129] have been found to boost mCRAMP expression.

1.3.2 Defensins

Defensins constitute another major family of AMPs in mammals. These are cationic peptides with a characteristic antiparallel β-sheet fold and consist of six highly conserved cysteine residues forming three pairs of disulfide bridges. Defensins, based on the size and on the alignment of the disulfide bonds are classified into α-, β-, and θ-defensins [130].

1.3.2.1 α- and θ- defensins

In human, there are six α-defensins: Human neutrophil peptides (HNP)-1 to 4 and human defensin (HD)-5 and -6. HNPs are abundant in PMNs and thus designated as neutrophil peptides. These peptides are also expressed in additional immune cells such as monocytes, NK cells, B cells and γδ T cells [103]. Paneth cells at the base of the small intestinal crypts are the main source of HD-5 and -6 [131, 132]. HD-5 has also been detected in female genital tract [133].

θ- defensins, identified only in non-human primates, are 18 residues long, cyclized peptides, with sequences closely related to the non-cyclized α-defensins [130].

Similar to cathelicidins, α-defensins (and also θ-defensins) are produced as preproprotein followed by removal of pre- and pro-sequences to generate active peptides [130].

1.3.2.2 β- defensins

Four β-defensins have so far been characterized in human: Human β-defensin (HBD)-1 to 4. HBD-1 was isolated from human blood filtrate [134] and HBD-2 and -3 were isolated from psoriatic scales [135, 136]. HBD-4 was identified, based on screening of genomic sequences and a synthetic peptide was made and functionally characterized [137]. However, natural HBD-4 has not yet been isolated. Twenty-eight additional genes encoding HBDs have been identified in the human genome [138]. In contrast to α- and θ-defensins, the precursors of β-defensins have a very short pro-sequence separating the signal and mature peptide regions [130].

HBDs are predominantly expressed in epithelial cells but also in leukocytes. HBD-1 is expressed constitutively in most tissues, while expression of HBD-2, -3 and -4 is often induced. However, HBD-1 expression can also be induced, for example by IFN-γ or by LPS in monocytes, macrophages and dendritic cells [139]. Induction of HBD-2 is also observed in these cells. Expression of HBD-2 and -3 is augmented in keratinocytes by *S. aureus* and IL-22 [124, 140]. In respiratory epithelial cells, HBD-2 expression is upregulated by *Pseudomonas aeruginosa*, tumor necrosis factor- α (TNF-α) and IL-1β [141]. TNF-α and heat-inactivated *P. aeruginosa* and *S. aureus* upregulate the expression of HBD-3 in primary keratinocytes and tracheal epithelial cells [136]. Expression of HBD-3 is also increased by the growth factors, IGF-1 and TGF-α, in keratinocytes [127]. HBD-4 expression is upregulated in primary respiratory epithelial cells after infection with *P. aeruginosa* and *Streptococcus pneumoniae* [137].

1.3.3 Functions of LL-37, β-defensins and their orthologues

1.3.3.1 Antimicrobial activity and microbial resistance

LL-37 is active against the gram negative bacteria *P. aeruginosa, Salmonella typhimurium, E.coli, Proteus mirabilis, Neisseria gonorrhoeae, N. meningitidis* [112, 126, 142, 143] and the gram positive bacteria *S. aureus, Staphylococcus epidermidis, Listeria monocytogenes* and GAS [106, 142]. LL-37 also displays inhibitory activity against the yeast *Candida albicans* [142] and some viruses [144-146]. The antibacterial activity of LL-37 positively correlates with the extent of helicity of the peptide, which in turn depends on the ionic environment and pH [85]. Helical content of LL-37 is enhanced with increasing concentration of salt and/or increasing pH and it adopts the α-helical conformation in physiological salt solution. However, Turner *et al.* have shown that certain organisms such as methicillin-resistant *S. aureus, P.mirabilis* and *C. albicans* are resistant to LL-37 at high salt concentration but are sensitive at low-salt media [142]. mCRAMP exhibits antimicrobial activity against GAS [106], uropathogenic *E. coli* [112], *N. meningitidis* [126] and vaccinia virus [146]. *S. typhimurium* survives and replicates inside macrophages derived from mCRAMP-deficient mice, but the replication is impaired in wild type macrophages [129]. *N. meningitidis* is sensitive to rCRAMP [147]. Rabbit CAP-18 exerts antimicrobial activity against *P. aeruginosa, E.coli, S. aureus*, methicillin-resistant *S. aureus*, *S. pneumoniae* and *S. typhimurium* [148, 149]. Moreover, LL-37 hampers the ability of uropathogenic *E.coli* to form curli-mediated biofilm [150].

HBD-1 displays antimicrobial activity against *P. aeruginosa* and *E.coli* [151]. HDB-2 is bactericidal for *P.aeruginosa, S.aureus* and antibiotic-resistant *E.coli, Klebsiella pneumoniae* and *P. mirabilis* [152]. HBD-3 is active against *P. aeruginosa, E. coli, Streptococcus pyogenes, S. aureus* (including multidrug-resistant strains) and vancomycin-resistant *Enterococcus faecium* [136]. *P. aeruginosa* and *Staphylococcus carnosus* are susceptible to HBD-4 [137]. Although all HBDs show antibacterial activities, the activity of HBD-1, -2 and -4 are inversely proportional to salt concentration [137, 151, 152], while HBD-3 is quite stable in a wide range of salt concentrations [136]. The formation of stable dimer or oligomer in solution has been suggested for the salt-resistance of highly cationic HBD-3 [153]. In addition to enhanced salt resistance, Midorikawa *et al.* have demonstrated that the antibacterial activity of HBD-3 against *S. aureus* is much stronger than that of HBD-1 or -2 [124]. However, platelet-derived HBD-1 displays potent antimicrobial activity against *S. aureus* and additionally induces PMNs to form NETs [154]. Recombinant HBDs possess antifungal activities against *Candida spp.* and the activity is more prominent for HBD-2 and -3 than HBD-1 [155]. Interestingly, after reduction of disulphide bridges, HBD-1 exerts potent antimicrobial activity against *C. albicans* and against anaerobic commensals of *Bifidobacterium* and *Lactobacillus* species [156]. Natural HBD-3 also impede *Candida albicans* [136]. HBD-2 and -3 inhibit HIV-1 replication in oral epithelial cells [157], while HBD-3 exhibits anti-viral activity against vaccinia virus [158] and prevent cervical epithelial cells from herpes simplex virus (HSV) infection [159]. Recombinant HBD-1 and -2 have antiparasitic activity against *Cryptosporidium parvum* sporozoites [160].

Synergistic or additive effects between HBDs and with LL-37 or other AMPs have also been documented. LL-37 and HBD-1 kill *H.pylori* individually and together these peptides reveal a synergistic effect [111]. LL-37 together with HBD-2 or -3 constitute synergistic effect against *S. aureus*, while the effect was additive with HBD-1 [124]. Combination of HBD-1, -2 and -3 shows additive effect against *S.aureus* [124]. HBD-4 exhibits synergy with lysozyme and strong additive effect with HBD-3 against *E. coli* and *S. carnosus* [137].

Several pathogenic microorganisms have evolved resistance to AMPs. Multiple strategies used by the pathogens to avoid antimicrobial effect of LL-37, HBDs and their orthologues are briefly discussed here.

(i) Pathogens can resist AMP activity by suppressing AMP expression. *Shigella* spp., *Vibrio cholera*e and enterotoxigenic *E. coli* (ETEC) downregulate LL-37 and HBDs in iEC [30, 31, 161]. LL-37 is also downregulated by *N. gonorrhoeae* in cervical epithelial cells [143]. Capsular *K. pneumoniae* impedes the expression of HBD-2 and -3 in airway epithelial cells [162].

(ii) Several bacteria such as *P. aeruginosa, P. mirabilis, Porphyromonas gingivalis*, *S. aureus, S. pyogenes* and *Enterococcus faecalis* secrete proteases that degrade LL-37 [163-166]. Secreted proteases from *P. mirabilis* and *P.gingivalis* also degrade HBD-1 and HBD-3, respectively [164, 167]. Cell-wall or cell-membrane associated proteases of enterohemorrhagic *E. coli* (EHEC) and *Yersinia pestis* cleave LL-37 [168, 169]. mCRAMP is degraded by cell membrane associated proteases of *Citrobacter rodentium* [170].

(iii) Secreted molecules other than proteases such as streptococcal inhibitor of complement (SIC) from *S. pyogenes* binds and traps LL-37 extracellularly [171].

(iv) Capsular polysaccharide (CPS) of *K. pneumoniae* limits the binding of HBD-1 and other AMPs to the bacterial surface [172]. CPS of *N. meningitidis* can resist LL-37, mCRAMP, HBD-1 and -2 [173, 174]. Notably, sublethal concentrations of AMPs have been demonstrated to augment transcription of the capsule genes [172-174]. Sequestration of HBD-3 and LL-37 by elaborated polysaccharide matrices of *S. epidermidis* also inhibits the antibacterial activity of these peptides [175, 176]. In addition, curli fimbriae of uropathogenic *E coli* bind and increase bacterial resistance to LL-37 and mCRAMP [150].

(v) Bacteria also resist AMP activation by modification of cell surface. Incorporation of D-alanine into teichoic acid of *S aureus* reduces negative charge of the cell wall and repels cationic peptides including mCRAMP [177]. Substitution of negatively charged cell membrane constituents of *S aureus* with cationic lysyl- phosphatidylglycerol confers resistance to LL-37 and HBD-3 [178]. Modification of lipid A head groups such as addition of 4-aminoarabinose (Ara4N) to the phosphate group in *P. aeruginosa* renders the pathogen resistant to HBD-1, HBD-2, LL-37 and CAP-18 [179]. *N. meningitidis* modifies lipid A head groups of lipooligosaccharide (LOS) with phosphoethanolamine and inhibits LL-37 interaction to the cell membrane [180]. Group B *Streptococcus* (GBS) can resist inhibitory activity of mCRAMP and LL-37 through cell surface-associated penicillin-binding protein 1a (PBP1a) without modifying bacterial surface charge or peptidoglycan cross-linking [181].

(vi) Removal of peptides by efflux pump makes *N. gonorrhea* and *N. meningitidis* resistant to LL-37 [180, 182]. Deletion of genes encoding AcrAB efflux pump in *K. pneumoniae* enhances bacterial sensitivity to defensins [183]. On the other hand, *H. influenza* imports LL-37 and HBD-3 by utilizing the Sap A (sensitivity to antimicrobial peptide A) transporter into the cytoplasm and subsequently degrades these peptides [184]. Interestingly, bacteria lacking a functional Sap permease complex accumulates AMPs in the periplasm of the outer membrane, whereas parental cells accumulate them in the cytoplasm and periplasm. SapA, thus protects cytoplasmic membrane from AMP activity [184].

(vii) Bacteria can exploit host systems for their resistance to AMPs. Attachment of *N. meningitidis* to epithelial cells and binding of polyamines to the outer surface of *N. Gonorrhea* contribute to their resistance to LL-37 [174, 185].

1.3.3.2 Additional functions

In addition to antimicrobial activity, numerous immunomodulatory and tissue homeostatic functions have been attributed to LL-37, HBDs and their orthologues. LL-37 exhibits chemotactic activity to PMNs, monocytes and T cells via binding to the formyl peptide receptor like-1 (FPRL-1) on the surface of these cells [103, 186]. mCRAMP also chemoattracts leukocytes by utilizing FPRL-1 or its mouse homologue formyl peptide receptor-2 [187]. HBD-1 displays chemotaxis of monocyte-derived dendritic cells (DCs) [188]. HBD-2 chemoattracts immature DCs and memory T cells through CCR6 receptor [189]. The chemotaxis of leukocytes by HBD-2 and -3 and their mouse orthologues MBD-4 and -14 involves CCR2 receptor [190]. LL-37 and HBD-2 display chemotactic activity to mast cells [191, 192]. Moreover, LL-37 and HBDs induce chemokine and cytokine secretion from different cell types [193]. These immunomodulatory functions may contribute to resolution of infection but may also give rise to inflammatory disorders. For instance, LL-37 can stimulate the synthesis of the proinflammatory lipid mediator leukotriene B4 from PMNs and form a positive feedback loop to perpetuate the inflammatory responses [194]. However, LL-37 and defensins also exert anti-inflammatory responses by direct binding and neutralization of microbial components, by inhibiting the production of pro-inflammatory mediators or by inducing anti-inflammatory cytokines [88, 193, 195-197]. In addition, rabbit CAP-18 was discovered as a LPS binding protein [89].

LL-37 mediates wound healing of skin and airway epithelia through inducing epithelial cell proliferation and migration [92, 95]. Intestinal epithelial barrier integrity is also maintained and reestablished by LL-37 [94]. LL-37 promotes cutaneous wound neovascularization through angiogenic activity, which involves increased proliferation of endothelial cells and subsequent vessel-like structure formation [93]. Similarly, HBD-2 stimulates chemotaxis, proliferation, capillary-like tube formation and enhances the speed of wound closure of human umbilical vein endothelial cells [91].

1.3.3.3 Physiological relevance

The physiological relevance of LL-37, HBDs and their orthologues has been demonstrated in human diseases as well as in animal models. An abundant expression of LL-37, HBD-2 and -3 is observed in psoriatic lesions of human skin compared to healthy skin, while in atopic dermatitis (AD), the induction is minor [122, 198]. The observed difference in the expression of these three AMPs is well correlated with the fact that eczemas of AD are highly susceptible to recurrent infections, while psoriatic lesions are rarely infected [199]. Moreover, a subset of AD patients with disseminated infection with HSV was demonstrated to exhibit significantly lower expression of LL-37, HBD-2 and -3 in skin biopsies compared to uncomplicated AD [200, 201]. The specific role of LL-37 orthologue in skin defense has been evaluated in mCRAMP knock-out (KO) mice. Compared to wild type mice ($Cnlp^{+/+}$), mCRAMP KO mice ($Cnlp^{-/-}$) are more susceptible to HSV, vaccinia virus and GAS infection [146, 200, 202, 203]. However, LL-37 has also been implicated in the pathogenesis of psoriasis. By forming complex with self-DNA or -RNA, LL-37 triggers abrupt activation of dendritic cells via toll-like receptors (TLRs) [204, 205]. LL-37 and its shorter forms are expressed abnormally in the facial skin of individuals with rosacea, stimulating skin inflammation [206].

Cystic Fibrosis (CF) is a lethal genetic disease caused by a mutation in the gene encoding CF transmembrane conductase regulator (CFTR). Patients with CF are susceptible to recurrent infections in airway surface epithelia (ASE) mainly by *P.aeruginosa* and *S. aureus*. CFTR dysfunction has been proposed to facilitate infections of ASE in several different manners [207]. One theory is the accumulation of excessive salt in airway surface fluid (ASF), leading to reduced antimicrobial activity of HBD-1 [151]. The antimicrobial activity of LL-37 is also compromised in CF lung fluids because of complex formation between LL-37 and glycosaminoglycans [208]. However, overexpression of LL-37/hCAP-18 in CF xenograft model was shown to restore the antimicrobial activity of the ASF [209]. Transgenic mice expressing LL-37/hCAP-18 in the respiratory tract was also demonstrated to decrease bacterial load and inflammatory responses following pulmonary challenge with *P. aeruginosa* [210]. Deletion of the gene encoding MBD-1in a mouse model of influenza infection was shown to stimulate an early inflammatory response in lung, leading to early lose of body weight and faster death of the mice [211].

The importance of LL-37 has also been exemplified in two rare congenital disorders. Patients with Morbus Kostmann, a neutropenic disease, are susceptible to recurrent infections and periodontitis. Treatment with granulocyte colony stimulating factor (GM-CSF) restores the level of PMNs but does not prevent infections, indicating functional deficiencies of PMNs. In fact, in a study by Putsep *et al.,* PMNs were found to be deficient in LL-37 and HNP1-3 [212]. In addition, LL-37 could not be detected in saliva and plasma of the patients [212]. Patients with neutrophil specific granule deficiency (SGD) also suffer from frequent bacterial infections. Low expression of LL-37 and other antimicrobial peptides/proteins may explain the susceptibility of these patients to infections [213].

The level of HBD-3 is significantly reduced in gingival crevicular fluid (GCF) of periodontitis patients, compared to healthy controls and is inversely proportional to bacterial colonization and the severity of the disease [214].

Systemic expression of LL-37/hCAP-18 in transgenic mice was shown to confer protection from induced sepsis [210]. Furthermore, rat receiving intraperitoneal LL-37 could prevent LPS-induced sepsis [215].

Low immunoreactivity of hCAP-18 in chronic skin ulcers of patients, as opposed to acute wounds reflects the physiological significance of LL-37/hCAP-18 in wound healing [92].

A combined effect of MBD-2 and -3 was shown in a mouse model of *P. aeruginosa* keratitis. Silencing MBD-2 or -3 individually led to increased bacterial load and elicited differential effects on disease pathology. However, silencing both genes elevated the bacterial load and inflammatory responses [216].

1.3.4 Induction of LL-37, β-defensins and their orthologues by extrinsic compounds

The expression of cathelicidins and defensins can be induced by different extrinsic compounds in a cell and tissue specific manner. Butyrate, a short chain fatty acid (SCFA), induces the expression of LL-37 preferentially in colonic epithelial cells [109, 217-220] and in lung epithelial cells [221]. Butyrate-induced LL-37 expression correlates with the increased antimicrobial activity of the colonic epithelial cell lysate [218]. Butyrate is a histone deacetylase inhibitor (HDACi), promoting acetylation of histones. Histone acetylation has been proposed for the induction of *CAMP* transcript by unwinding the chromatin structure, facilitating binding of transcription factors at the promoter region [220, 221]. In agreement with this, a synthetic HDACi, trichostatin A (TSA) induces *CAMP* expression [220]. Binding of transcription factors such as vitamin D receptor (VDR), putative activator protein 1 (AP-1) and PU.1 of the Ets family to the corresponding binding sites at the promoter region has been demonstrated for butyrate-induced expression of *CAMP* gene [219, 221, 222]. Furthermore, Inhibition of different mitogen-activated protein kinases (MAPK) hinders *CAMP* induction by butyrate [217, 219, 221]. Butyrate also induces HBD-2 in monocytes [218].

Our group has found that phenylbutyrate, an analogue of butyrate upregulates the expression of the *CAMP* gene in different epithelial cell lines and also in monocytes [223]. The involvement of MAPK signaling cascade was demonstrated for this induction. However, PB could not mediate significant changes in histone acetylation at the *CAMP* proximal promoter, although an increase in genome-wide histone acetylation was observed. Interestingly, inhibition of protein synthesis by cycloheximide (CHX) blocked PB and butyrate mediated *CAMP* gene induction. These results indicate no direct effect of PB on the chromatin structure at the *CAMP* gene promoter. Rather, enhanced histone acetylation indirectly induces the *CAMP* gene by promoting expression of genes, encoding critical regulatory factors. Gene encoding HBD-1 was also induced by PB in lung epithelial cells, but was downregulated in PB-stimulated monocytes [223].

The hormonal form of vitamin D3 (1, 25- dihydroxyvitamin D3) and its analogues upregulate the *CAMP* gene expression in keratinocytes, oral/ lung/colonic epithelial cells, monocytes, PMNs, bone-marrow (BM) derived macrophages and fresh BM cells [224, 225]. Gene encoding HBD-2 is also induced by vitamin D in

keratinocytes and in oral/ lung epithelial cells [224]. The induction is mediated through interaction between VDR and a consensus vitamin D response element (VDRE) in the promoter of the *CAMP* gene [224, 225]. Since, VDRE is absent in the *Cnlp* gene, LL-37 induction by vitamin D does not occur in murine cells [225]. A synergistic effect of LPS and vitamin D was observed on *CAMP* gene expression in PMNs, which correlated with increased antimicrobial activity of PMNs [224]. In human macrophages, activation of TLRs upregulates VDR and the vitamin D_1 hydroxylase genes, leading to the induction of LL-37 with subsequent killing of intracellular *Mycobacterium tuberculosis* [226]. Interestingly, tuberculosis susceptible African-American individuals have low serum levels of 25-hydroxyvitamin D3 and hence are less efficient in inducing the expression of LL-37 [226].

Butyrate and vitamin D have tissue specific preference for colonic epithelial cells and keratinocytes, respectively [218]. However, butyrate or TSA can amplify the vitamin D-induced expression of LL-37 in keratinocytes via increased acetylation of histones [227]. A synergistic effect of PB and vitamin D was also observed on *CAMP* gene expression in lung epithelial cells [223].

L-isoleucine promotes HBD-2 expression in pulmonary epithelial cells [228]. In lung extract of *M. tuberculosis* infected mice, significant induction of MBD-3 and -4 after intratracheal administration of L-isoleucine was demonstrated to correlate with decreased bacillary loads and tissue damage [228].

Additional components such as lithocolic acid (LCA) and trace metal zinc induce LL-37 expression in colonic epithelial cells and LCA was shown to have additive effect with butyrate [222, 229].

1.4 BUTYRATE IN INTESTINAL INFLAMMATION

Short chain fatty acids (SCFAs), particularly acetate, propionate and butyrate are produced through fermentation of mainly undigested dietary carbohydrates and other dietary and endogenous substrates by the colonic flora [230, 231]. SCFAs, predominantly butyrate are rapidly absorbed and metabolized by the colonic mucosa and provide energy for colonocytes [231]. Butyrate exerts various additional effects, influencing colonic health such as proliferation and differentiation of colonocytes, epithelial barrier function, transepithelial ion transport, visceral perception and intestinal motility. Moreover, butyrate affects regulation of immunity, inflammation and oxidative stress [230]. Several extra-intestinal effects of butyrate have also been reported [230].

The role of butyrate in intestinal inflammation has been investigated in several intervention studies on patients with inflammatory bowel disorders. In a randomized, single-blind controlled trial, administration of butyrate enema in patients suffering from distal ulcerative colitis (UC) resulted in an improvement in clinical, endoscopic and histologic features [232]. In an open-labeled study, patients with distal UC, refractory to available rectal therapy and to oral sulfasalazine or mesalamine (also known as mesalazine or 5-ASA), exhibited 40% complete response rate and an overall 60% response rate after receiving butyrate enemas [233]. In a randomized, double-blind, placebo-controlled trial, Vernia *et al.* found a significantly better improvement of disease activity index (DAI) and overall clinical index of UC after treatment with

combination of oral sodium butyrate and oral mesalazine compared to mesalazine treatment alone [234]. The same group later demonstrated a similar effect of topical administration of butyrate together with oral mesalazine in the treatment of UC [235]. In a randomized, double-blind, cross-over trial, significant remission of clinical score and majority of individual clinical, endoscopic and histological parameters of acute radiation proctitis (ARP) was observed after topical treatment with butyrate but not with saline [236]. When the treatment regimen was switched to butyrate, the majority of the previously placebo-treated patients had remedial of symptoms. On the other hand, one-third of the previously butyrate-treated patients exhibited a relapse of ARP symptoms, when switched to saline [236]. In another study, significant decrease in DAI of UC by butyrate enema correlated with significant reduction of PMNs in crypt and surface epithelia as well as lymphocytes/plasma cells in lamina propria (LP) of the rectal mucosa [237]. Interestingly, nuclear translocation of nuclear factor κβ (NF-κβ) was observed in almost all LP macrophages before treatment, which was reduced after butyrate treatment. These findings indicate that butyrate exert anti-inflammatory response in UC, in part by reducing production of inflammatory mediators through inhibition of NF-κβ activation [237]. Patients with mildly to moderately active Crohn's disease (CD) experienced significant improvement in DAI, endoscopical and histological score, and significant reduction of mucosal level of NF-κβ and IL-1β after receiving enteric-coated butyrate tablets [238]. When colonic biopsy specimens and isolated lamina propria mononuclear cells (LPMC) from CD patients were cultured with butyrate, reduction of proinflammatory cytokines and inhibition of nuclear translocation of NF-κβ was observed [239]. Similar effect of butyrate was observed in LPS-stimulated peripheral blood mononuclear cells (PBMC) [239].

In an animal model of chemically-induced colitis, clinical recovery, improvement of colonic damage score and electrolyte absorption after butyrate enema treatment correlated with reduced myeloperoxidase (MPO) activity[240]. Furthermore, IFNγ/STAT1 signaling, which plays an important role in the pathogenesis of IBD, was inhibited by butyrate in colonic epithelial cell lines [241].

2 RATIONALE, HYPOTHESIS AND AIMS OF THE STUDY

The emergence of multidrug-resistant bacterial strains due to uncontrolled use of antibiotics is a serious threat for the management of infectious diseases. Adverse effects of antibiotic treatment such as disruption of the normal gut flora and release of toxic microbial components further worsen the situation. Therefore, development of novel antimicrobial therapies is warranted. AMPs with a broad spectrum of antimicrobial activity complemented with immunomodulatory activities are attractive therapeutic candidates. AMPs are bactericidal rather than bacteriostatic and in contrast to traditional antibiotics, attack multiple targets within the bacteria, leading to low propensity for the development of bacterial resistance (reviewed in [242]). Although, pathogens have developed mechanisms to resist AMP activity, the resistance appears to be modest compared to conventional antibiotics [242]. Moreover, AMPs can be utilized as adjunct therapy to current antibiotics in a synergistic or additive manner. The role of AMPs in tissue homeostasis such as angiogenesis and wound healing may also be exploited, when using these peptides as anti-infective drugs.

Utilizing AMPs as template, antimicrobial drugs have been synthesized. However these drugs have certain drawbacks for therapeutics usage. In addition to high production cost, systemic delivery of these drugs is limited by poor pharmacokinetics, due to their susceptibility to proteases and unknown toxicity profile against the host cells [242]. Since peptide-based drugs are unlikely to be resorbed from the gastrointestinal tract, oral administration is also not feasible. Although, several approaches have been made to overcome these limitations, to date only few of these drugs have undergone clinical trials [242]. Most of the drugs in clinical trials, have been restricted to topical applications and only gramicidin S and polymyxin B, which are based on bacterial AMPs, have been approved so far by FDA for topical use.

A promising alternative option would be the induction of endogenous AMPs by topical or systemic administration of extrinsic compounds. With the advent of tissue preferring stimulants, it is possible to induce abundant amount of AMPs at the sites of infection without systemic accumulation, reducing the risk of adverse systemic reactions. It is also possible to stimulate a battery of AMPs by using very small number of stimulants, making the approach more feasible than delivering several AMPs together. Inducers, such as butyrate, vitamin D, zinc, LCA and isoleucine are synthesized by the host itself or obtained from the diet and are required for physiological functions. These natural compounds or their analogues are readily available at low cost, making this strategy cost-effective and relatively safe. Phenylbutyrate is even a FDA- approved drug for urea cycle disorder.The induction approach may particularly be important, when pathogens downregulate AMPs at the local site of infection as a means of immune evasion.

In a previous study, our group found that the expression of LL-37 and HBD-1 is downregulated in large intestinal epithelial cells of patients, during acute shigellosis, thereby facilitating bacterial invasion [30]. We hypothesized that this downregulation could be counteracted via induction of AMPs by using butyrate or phenylbutyrate. This counteraction would correlate with the improved clinical, histological and microbiological features of shigellosis, as demonstrated in a rabbit model by colonic infusion of SCFA mixture (acetate, propionate and butyrate) [243]. Since, patients with

shigellosis are often prone to secondary respiratory infections [2-4], we assumed that *Shigella* infection would also reduce AMP expression in respiratory epithelia, which could be counteracted by treatment with butyrate or phenylbutyrate. As antibiotics exhibit different immunomodulatory effects [244], the expression of AMPs might also be affected by antibiotics.

The aims of the thesis were:

I. To investigate the effect of oral administration of sodium butyrate (NaB) in a rabbit model of shigellosis on clinical outcome, intestinal inflammation, bacterial load in stool, the expression of CAP-18 in large intestinal mucosa and the processing of CAP-18 in stool.

II. To evaluate the efficacy of NaB enema as adjunc therapy to antibiotics in patients with shigellosis by analyzing clinical, histopathological and microbiological outcome, the expression of LL-37 in large intestinal mucosa and the levels of released LL-37, HBD-1 and HBD-3 in stool.

III. To study the effect of oral sodium-4-phenylbutyrate (PB) treatment in a rabbit model of shigellosis on clinical consequences, bacterial shedding in stool, CAP-18 expression in the large intestinal mucosa and the processing of CAP-18 in stool. Moreover, the effect of PB and NaB on CAP-18 expression in lung and tracheal mucosa were examined.

IV. To determine, whether antibiotics affect the expression of AMPs in colonic epithelial cells *in vitro* and *in vivo* and hence contribute to the antibiotic-associated diarrhea caused by *Clostridium difficile.*

3 METHODOLOGY

The methods utilized in this thesis are listed below. Detailed description of the methods can be found in the papers as referred.

1) Animal model (rabbit model of shigellosis)	: Paper I, III, IV
2) Administration of NaB/saline in patients	: Paper II
3) Clinical investigations and endoscopic evaluation of inflammation in the rectal mucosa of patients	: Paper II
4) Biopsy collection and processing for histology/immunohistochemistry	: Paper I, II, III, IV
5) Bacterial counts in stool	: Paper I, II, III
6) Inhibition zone assay (antibacterial assay) and depletion of antibacterial activity	: Paper I
7) *In vitro* bacterial killing assay	: Paper I, IV
8) Stool extraction	: Paper I, II, III
9) Cell culturing (HT-29) and stimulation	: Paper IV
10) Histone extraction	: Paper IV
11) Enrichment of peptide/protein	: Paper I, III, IV
12) Western blot analysis	: Paper I, III, IV
13) Real-time PCR	: Paper I, III, IV
14) Microarray analysis	: Paper IV
15) Histology	: Paper I, II
16) Immunohistochemistry	: Paper I, II, III, IV
17) Image analysis	: Paper I, II, III, IV (Paper III supporting information S1 for details)
18) Immunofluorescence staining	: Paper IV
19) Enzyme-linked immunosorbent assay (ELISA)	: Paper II
20) Measurement of butyrate levels in blood	: Paper III
21) Biosafety evaluation of NaB or PB treatment	: Paper I, II,III

4 RESULTS AND DISCUSSION

4.1 CATHELICIDIN INDUCTION BY BUTYRATE OR PHENYLBUTYRATE TREATMENT CORRELATES WITH IMPROVED CLINICOPATHOLOGICAL AND MICROBIOLOGICAL FEATURES OF SHIGELLOSIS (PAPER I-III).

In paper I, a non-surgical rabbit model of shigellosis was developed according to Etheridge *et al.* [245] to investigate the therapeutic potential of sodium butyrate (NaB). Rabbits infected with *S. flexneri* 2a developed human-like dysenteric symptoms such as thick liquid stool with mucus and occasional blood, decreased body weight, transient fever, lethargy and anorexia, usually within 24 hours of infection. Hence, this experimental model of shigellosis was suitable for therapeutic intervention. The efficacy of NaB as adjunct therapy to antibiotics was then evaluated in adult patients with shigellosis in a double-blind, randomized clinical trial (paper II). However, oral administration of NaB in human is limited by its characteristic bad smell. Therefore, in paper III, we investigated the therapeutic effect of sodium-4-phenylbutyrtae (PB), an odorless butyrate analogue in the same rabbit model of shigellosis.

4.1.1 Effect of butyrate or phenylbutyrate treatment on the expression of cathelicidins and β-defensins in gut mucosa and lumen during shigellosis (paper I-III)

By utilizing immunostaining technique, CAP-18 peptide/protein (designated for mature CAP-18 peptide/proform) expression was largely detected in the surface epithelia (SE) of colonic and rectal mucosa in healthy rabbits (paper I and III). The expression was very low in lamina propria (LP). When the rabbits were infected with *Shigella*, a significant downregulation of CAP-18 peptide/protein was observed in colonic and rectal SE. However, numerous LP cells were positive for CAP-18 peptide/protein after *Shigella* infection. These results confirm our previous findings, where we showed downregulation of LL-37/hCAP-18 in the epithelia and influx of LL-37/hCAP-18 positive PMNs and macrophages in LP of the rectal mucosa in patients with acute shigellosis [30]. In that study, suppression of the *CAMP* gene and the gene encoding HBD-1 in gut biopsies and in colonic epithelial and monocytic cell lines was also demonstrated. Bacterial plasmid DNA was indicated as the potential mediator of this downregulation [30]. Reduced expression of the *CAMP* gene and the gene encoding HBD-3 in *Shigella*- infected colonic epithelial cells was also reported in a later study [31]. The authors suggested a set of virulent plasmid-encoded effectors, regulated by the MxiE transcriptional activator, being involved in this downregulatory process.

Oral treatment of *Shigella*-infected rabbits with NaB restored CAP-18 peptide/protein expression in colonic and rectal SE (paper I). On the other hand, CAP-18 peptide/protein positive cells in LP were decreased considerably after NaB treatment, suggesting a low influx of inflammatory leukocytes. Similar to NaB treatment, oral PB treatment counteracted CAP-18 peptide/protein expression in the rectal SE (paper III). In addition to epithelial induction, NaB or PB treatment prompted the secretion and/or processing of CAP-18 in intestinal lumen. Western blot analysis of stool extracts revealed CAP-18 proform in healthy and majority of the infected rabbits.

NaB treatment resulted in the appearance of processed CAP-18 peptide in stools, collected every day during the treatment period (paper I). The proform was also present in the beginning of the treatment, which disappeared later. By PB treatment, the level of proform was elevated and low level of processed CAP-18 peptide was detected throughout the treatment regimen (paper III). Thus, we speculated that NaB or PB might activate some proteases that cleaved the CAP-18 precursor to generate the mature peptide in the gut. The processing enzyme has not been identified so far. However, in a recent microarray analysis (paper IV, not published), we found an induction of the gene encoding kallikrein-1 in NaB-stimulated colonic epithelial cell line. Kallikerin-5 was previously shown to process hCAP-18, generating LL-37 and several other truncated peptides in keratinocytes [118]. Mature CAP-18 peptide was also detected in the stool of one infected rabbit, which most probably originated from the infiltrating immune cells. The size difference between peptides derived from the infected rabbit and the treated rabbits might reflect the different processing of the precursor proteins in different cell types (epithelial or immune cells). Further studies will be needed to validate this hypothesis.

Low expression of LL-37/hCAP-18 in the rectal epithelia was observed in *Shigella*- infected patients at the acute stage (day 1) (paper II). However, ample level of LL-37/hCAP-18 was detected in stool extract by ELISA on day 1, which was consistent with the high frequency of inflammatory cells in stool. Administration of NaB as enema significantly augmented LL-37/hCAP-18 expression in the rectal epithelia on day 7 compared to that in placebo treated patients. Levels of LL-37/hCAP-18 in stool went down significantly in both treatment groups from day 4 onwards, reflecting recovery from infection and consequent reduction of inflammation due to antibiotic treatment (discussed later). However, concentration of LL-37/hCAP-18 in stool remained significantly higher on day 4 and 7 in NaB-treated patients compared to placebo-treated patients. Large intestinal epithelia could be the source of LL-37/hCAP-18 during the recovery phase of the disease. No difference was eminent in the levels of HBD-1 and HBD-3 in stool between the NaB- and placebo- treated patients throughout the study period.

4.1.2 *Shigella* count in the gut lumen after treatment with butyrate or phenylbutyrate (paper I and III)

In the previous study, we proposed that the downregulation of AMPs in the epithelial surface of the large intestine may facilitate survival of *Shigella.* In fact, LL-37 peptide was shown to exert very efficient *in vitro* bactericidal activity against *S. dysenteriae* type I*, S. flexneri* and *S.boydii* in that study [30]. The *in vivo* significance of mCRAMP in reducing colonization of colonic epithelia by *Citrobacter rodentium* and subsequent systemic infection was shown in KO mice [246]. Furthermore, Sperandio *et al*. demonstrated that wild type *S. flexneri* strain capable of downregulating HBDs and LL-37 in the colonic epithelial cells, progresses deeply in the mucosal layer towards intestinal crypt of a xenotransplant model [31]. When *Shigella*-infected rabbits were treated with NaB or PB, *Shigella* load in stool declined gradually over time. Release of mature CAP-18 in the gut lumen suggested the involvement of this peptide in shigellacidal activity, although there might be additional antimicrobial components in the the lumen. Indeed, in inhibition zone assay, stool extract of a NaB- treated rabbit

exhibited antibacterial activity against *E. coli* strain D21 and 20% of this activity was blocked with CAP-18 specific antibody but not with an unspecific IgY antibody (paper I). In line with this, 97-99% killing of *S. dysenteriae* type I and *S. flexneri* 2a was observed *in vitro* with low concentration of synthetic CAP-18 peptide (0.9 μM and 1.13 μM, respectively). Interestingly, in the presence of 40 mM NaB, lower amount of CAP-18 peptide were required for achieving similar killing efficiency against these two strains (0.68 μM and 0.9 μM, respectively). NaB alone could also kill both strains of *Shigella*, but at a very high concentration (400 mM). Thus, NaB (40 mM) worked with CAP-18 peptide in an additive manner to kill off *Shigella*.

4.1.3 Clinical responses in shigellosis after treatment with butyrate or phenylbutyrate (paper I-III)

Counteraction of *Shigella*-mediated downregulation of CAP-18 peptide/protein in gut epithelia and reduction of bacterial load in lumen by treatment with NaB or PB, correlated with the clinical recovery from shigellosis in the rabbit model (paper I and III). Infected rabbits treated with saline never recovered from the disease and several of them died within 48 to 72 hours of infection. Two rabbits died even before the start of the treatment. Bacteremia and consequent septic shock could be the cause of death of these rabbits. All rabbits survived after treatment with NaB or PB and recovered from the disease within 3-5 days. Reduction of liquid stool and red blood cells (RBC) in stool was noticeable after 24 hours and maximum recovery was achieved within 72-96 hours of treatment. However, rabbits continued to lose body weight until 48 hours after infection and then started to recover. Rabbits also revived from lethargy and anorexia after treatment.

In clinical trial, *Shigella* count in stool disappeared within 48 hours in all patients and no significant difference was observed in terms of clinical recovery between NaB- and placebo-treated patients (paper II). Since, all patients were undergoing antibiotic treatment, simultaneous diminution of bacterial shedding and consequent clinical responses in both treatment groups were not unexpected.

4.1.4 Inflammatory responses in shigellosis after treatment with butyrate or phenylbutyrate (paper I-III)

Reduction of CAP-18 peptide/protein positive inflammatory cells in large intestinal LP of NaB-treated rabbits demonstrated anti-inflammatory effect of the treatment (paper I). No obvious difference in CAP-18 peptide/protein expression in LP was observed between infected and PB-treated rabbits. Although, in patients, the efficacy of NaB adjunct therapy on bacteriological and clinical recovery could not be evaluated, the effectiveness on the reduction of inflammatory responses was manifested (paper II). Sigmoidoscopic examination of the rectal mucosa revealed healed/reduced inflammation on day 7 after NaB treatment compared to day 1 in 11 out of 15 patients (73.3%). Notably, 9 patients (60%) with mild (n=4), moderate (n=2) or severe (n=3) inflammation on day 1 had completely healed mucosa on day 7. In contrast, in placebo-treated group, 6 out of 11 patients (54.5%) showed reduction of rectal inflammation on day 7 and complete healing was observed only in 4 patients (36%), who presented mild (n=3) or moderate (n=1) inflammation on day1. By histological analysis of the rectal

biopsies, decrease of inflammation from day 1 to day 7 was noticed in 92.8% (13 out of 14) patients in NaB- treated group in contrast to 50% (5 out of 10) patients in placebo-treated group, showing statistically significant difference. In NaB-treated group, normal histology was observed in day 7 biopsies from 11 patients (78.6%), who had severe, moderate or mild inflammation (n= 1, 3 and 7, respectively) on day 1. In placebo-treated group, only one patient (10%) was completely recovered from histological features of inflammation on day 7, which was significantly lower compared to NaB-treated group. The diminished retrieval of mucosal inflammation in placebo-treated patients was in agreement with our previous findings, where persistence of proinflammatory cytokines and immune cells in the large intestinal mucosa were demonstrated even after clinical recovery from shigellosis [23, 25]. Moreover, an earlier reduction of macrophages and pus cells in stool was observed (by day-2) in NaB-treated patients than that in the placebo-treated patients. Although the levels of proinflammatory cytokines, IL-8 and IL-1β in stool declined over time in both treatment groups, the reduction was higher in NaB-treated patients compared to placebo-treated patients.

Eventual reduction of macrophages, pus cells and proinflammatory cytokines in both groups of patients indicated that the *Shigella*-mediated inflammatory responses were reduced with the recovery from infection due to antibiotic treatment. In the rabbit model, NaB treatment would decrease the inflammatory responses through reduction of *Shigella* load in the gut lumen. However, earlier decline of mucosal and luminal inflammation in NaB-treated patients suggested additional anti-inflammatory effects of butyrate. Indeed, butyrate was shown previously to reduce the inflammatory responses in UC, CD and other inflammatory bowel disorders in patients and also in animal model of colitis [232-241]. Induced LL-37 and CAP-18 peptide could also contribute to the lessening of inflammation by neutralizing *Shigella* components, by inhibiting production of proinflammatory mediators or by stimulating anti-inflammatory cytokines [88, 89, 193, 195, 196]. Butyrate, by stimulating colonocyte proliferation [247], and LL-37/CAP-18 peptide, by wound healing and angiogenic activities [92, 93, 95] might facilitate healing of epithelial erosion and mucosal damage during severe shigellosis.

Overall, treatment with NaB or PB resulted in the induction of epithelial expression of LL-37/hCAP-18 or rabbit CAP-18 peptide/protein, secretion of mature active peptide in the lumen along with reduction of luminal *Shigella* load and intestinal inflammation, and recovery from clinical symptoms of shigellosis. The improvement of clinical, bacteriological and pathological features of shigellosis was in line with the previously demonstrated effects of SCFA mixture [243]. Interestingly, in a recent randomized clinical trial, ingestion of green banana was shown to increase luminal SCFAs levels in *Shigella*-infeced children, which correlated with clinical recovery and reduced severity of inflammation [248].

4.1.5 Effect of butyrate or phenylbutyrate treatment on the expression of CAP-18 peptide/protein in the mucosa of respiratory tract (paper III)

In the rabbit model of shigellosis, CAP-18 peptide/protein was also downregulated in the epithelia of lung and trachea, indicating a systemic effect of *Shigella* infection. Suppression of innate effector molecules during shigellosis could lead to deficient barrier function of the respiratory tract, facilitating invasion by opportunistic respiratory pathogens. In fact, earlier studies reported that children with shigellosis and persistent diarrhea are often susceptible to secondary respiratory infections such as pneumonia with a fatal outcome [2-4]. Interestingly, treatment with NaB or PB counteracted the downregulation of CAP-18 peptide/protein in lung epithelia, suggesting the role of these components in restoring chemical defense barrier of the lung. Butyrate was shown earlier to disseminate into blood after oral administration of NaB to mice and rats [249]. We could also detect butyrate in serum in particular at 30 minutes after oral treatment of healthy rabbits with a single dose of NaB. This finding indicated that orally administered NaB (or PB) after being absorbed in the intestine, reached the respiratory mucosa through the blood stream. Induction of CAP-18 peptide/protein expression in the epithelia of lung, rectum and colon after intravenous injection of *Shigella*-infected rabbits with NaB further supported the systemic distribution of these components.

4.1.6 Effect of butyrate or phenylbutyrate treatment on the expression of CAP-18 transcript in the mucosa of large intestine and respiratory tract (paper I and III)

In parallel to CAP-18 peptide/ protein expression, we also measured CAP-18 transcript in the biopsy specimens of colon, rectum, lung and trachea utilizing real-time qPCR. In the first study (paper I), extracts of colonic and rectal biopsies contained all layers of mucosa. Expression of CAP-18 transcript was very high in *Shigella*-infected rabbits compared to healthy rabbits. Immunohistochemical detection of CAP-18 peptide/protein positive immune cells in LP during infection indicated that these recruited cells were the source of elevated transcript level. This hypothesis was further supported by the presence of highest level of CAP-18 transcript in tissues with most severe histological grade of inflammation. Accordingly, healing of the intestinal inflammation with NaB treatment coincided with the drop of CAP-18 transcript to the level of healthy controls. To elucidate the spatial distribution of CAP-18 transcript in the large intestinal mucosa, epithelial and LP cells were separated in the later study (paper III). Expectedly, CAP-18 transcript was augmented significantly in LP cells of the the gut mucosa after infection compared to healthy rabbits. However, in contrast to the peptide/protein level, CAP-18 transcript in epithelial cells of the colon was enhanced after *Shigella* infection, indicating a possible post-transcriptional regulation. Low expression of proteins despite ample accumulation of corresponding transcripts was also noticed earlier for several cytokines in the rectum of patients with shigellosis [250]. In support to these findings, Hale *et al.* demonstrated inhibition of protein synthesis in epithelial cell lines, infected with *S. dysenteriae* 1 or *S. flexneri* [251]. Shiga toxin or shiga-like cytotoxins were shown to be responsible for this effect. After initial infection, host probably produces high level of CAP-18 mRNA to strengthen the

innate defense against the infecting pathogens. However, pathogens may in someway interrupt translation to escape host defense.

Upregulation of CAP-18 transcript in the gut mucosa of rabbit after *Shigella* infection was opposite to our earlier finding in patients with shigellosis [30]. In that study, we demonstrated downregulation of the *CAMP* gene in gut biopsies at the acute stage of disease (day 1, enrolment day), and the proportion of patients with this downregulation increased in the early recovery phase (day 11 and day 30 after enrolment for adult and child patients, respectively). The acute stage generally refers to 3-5 days after the onset of diarrhea. Therefore, we could not evaluate the fate of the *CAMP* gene in patients during the very early phase of infection. On the other hand, the infected rabbits could not be kept alive without treatment for longer periods (e.g. a week) to measure CAP-18 transcript at later phase. Moreover, patients were under treatment with antibiotics. In a recent study (paper IV, unpublished), we showed that ciprofloxacin suppresses butyrate-induced expression of the *CAMP* gene in colonic epithelial cell lines within 24-48 hours of stimulation. Hence, antibiotic treatment might also explain the decrease of the *CAMP* gene in gut biopsies of patients.

The upsurge of CAP-18 transcript after *Shigella* infection was also observed in the mucosa of lung and trachea. NaB or PB treatment normalized the *Shigella*-mediated accumulation of CAP-18 mRNA in intestinal and respiratory mucosa, while amplified peptide/protein expression in the epithelia. The detailed mechanisms of this post-transcriptional regulation need to be elucidated.

4.1.7 Safety profile of butyrate or phenylbutyrate treatment (paper I-III)

To evaluate the safety profile of oral NaB or PB treatment in rabbits and humans, the renal (creatinine and urea) and hepatic (alanine transaminase and gamma glutamyl transferase) biomarkers for toxicity were assessed. Treatment of healthy rabbits with various doses of NaB did not affect the plasma levels of these biomarkers (paper I). A similar outcome was observed when healthy rabbits were treated with PB (paper III). Notably, levels of creatinine, urea and alanine transaminase were increased after *Shigella* infection, which was normalized after PB treatment. These findings demonstrated no adverse effects of oral NaB or PB treatment in rabbits, rather PB treatment reduced the *Shigella*-mediated systemic toxicity. In patients, the serum levels of urea, alanine transaminase, gamma glutamyl transferase were within the normal range after topical NaB treatment (paper II). The level of creatinine was marginally below the normal range in both treatment groups. Previous intervention studies also did not report any adverse side effects of oral (2-4 g /day for 6-8 weeks) or topical (80-100 mM/ day for 6-8 weeks) butyrate treatment in patients with UC and CD [234, 235, 237, 238]. Moreover, application of NaB enema (80 mM, twice daily for 4 days) in healthy adults did not induce histological changes in the rectal mucosa [247]. PB is a FDA approved drug for urea cycle disorder and is well tolerated with minimum side effects (www.drugs.com/cons/sodium-phenylbutyrate.html).

4.2 ANTIBIOTIC-MEDIATED SUPPRESSION OF ANTIMICROBIAL PEPTIDE INDUCTION IN COLONIC EPITHELIAL CELLS: IMPLICATION FOR *CLOSTRIDIUM DIFFICILE* ASSOCIATED DIARRHEA (PAPER IV)

4.2.1 Effect of antibiotics on butyrate-induced expression of cathelicidins and β-defensins in colonic epithelial cells

Apart from exhibiting bactericidal or bacteriostatic effects on pathogens, antibiotics may alter bacterial properties to facilitate efficient clearance by the host immune system [252]. On the other hand, release of bacterial components such as LPS, teichoic and lipoteichoic acids, peptidoglycan, DNA and proteins due to antibiotic treatment can induce proinflammatory responses in host cells [253]. In addition, several antibiotics directly modulate expression and functions of both innate and adaptive immune responses [244]. For instance, fluoroquinolones, a class of antibiotics attenuate proinflammatory cytokine responses *in vitro* and *in vivo*, exerting beneficial effects on clinical response [254]. Ciprofloxacin, a fluoroquinolone antibiotic, increases the phagocytosis or killing capacity of PMNs and macrophages [255, 256]. Expression of innate immune effectors such as prostaglandin E2, nitric oxide and receptors such as TLRs and CD-14 are also modulated by ciprofloxacin [257, 258]. In this study, we demonstrated the immunomodulatory effects of antibiotics on the expression of AMPs.

We showed that ciprofloxacin dose-dependently suppresses butyrate-induced expression of LL-37 transcript and LL-37/hCAP-18 in the colonic epithelial cell line HT-29 after 24 or 48 hours of stimulation. However, cells stimulated only with ciprofloxacin did not show any obvious changes in the expression of the *CAMP* gene or LL-37/hCAP-18. In fact, quinolone antibiotics generally do not exhibit detectable immunomodulatory activities in the absence of co-stimulant such as endotoxins, cytokines or stress [259]. Moreover, induction of LL-37 or rabbit CAP-18 in colonic epithelial cells is physiologically relevant, as butyrate is constantly produced in the large intestine by the fermentation of dietary substrates [230, 231]. The *in vivo* expression of CAP-18 peptide/protein in the rectal epithelia of healthy rabbits was also suppressed by ciprofloxacin as revealed by immunostaining. In addition, in the rabbit model of shigellosis, ciprofloxacin inhibited butyrate-induced expression of CAP-18 peptide/protein. Moreover, induction of HBD-3 but not HBD-1 transcript by butyrate was repressed by ciprofloxacin in HT-29 cells. No effect of NaB or combination of NaB and ciprofloxacin was observed on the expression of HBD-2 transcript.

The *in vitro* and *in vivo* effects of ciprofloxacin were compared with pivmecillinam, a β-lactam antibiotic. Pivmecillinam did not exert any effect on the expression of the *CAMP* gene or LL-37/hCAP-18 in HT-29 cells. However, downregulation of CAP-18 peptide/protein was observed in the rectal epithelia of healthy rabbits after pivmecillinam treatment. These findings pointed to the *in vivo* activation of pivmecillinam, which is actually an orally-active prodrug. The effect of pivmecillinam on CAP-18 peptide/protein expression in healthy rabbits was weaker compared to ciprofloxacin and in *Shigella*-infected rabbits treated with NaB, it did not elicit any obvious effect.

To further compare between fluoroquinolones and β-lactam antibiotics, two additional antibiotics in each group were assessed. β-lactam ampicillin and ceftriaxone had no effect on butyrate-induced *CAMP* gene expression. On the other hand, ofloxacin and levofloxacin, antibiotics of the fluoroquinolone group inhibited the *CAMP* gene induction, but the effect was not significant. These two fluoroquinolone antibiotics are structurally very similar to each other but different from ciprofloxacin. While, ciprofloxacin possesses a cyclopropyl ring at position N1 of the quinolone ring, ofloxacin and levofloxacin lack this ring [259]. Earlier, it was shown that, quinolones having cyclopropyl ring display enhanced anti-leukemic and hematopoietic effects, as opposed to the quinolones lacking this ring [260, 261]. Presence of cyclopropyl ring might explain the stronger suppression of butyrate-induced *CAMP* gene expression by ciprofloxacin compared to ofloxacin and levofloxacin.

Antibiotics of other classes such as clindamycin (a lincosamide antibiotic), azithromycin (a macrolid antibiotic) and isoniazid (organic compound and anti-tuberculosis drug) were also evaluated for the effect on *CAMP* gene induction. Only clindamycin exhibited significant suppression, but the effect was less prominent than ciprofloxacin. While azithromycin non-significantly inhibited butyrate-induced *CAMP* gene expression, isoniazid exerted no effect.

Overall, variable effects on cathelicidin induction were observed between antibiotics from different classes and even between antibiotics from the same class; only ciprofloxacin and clindamycin displayed significant effects.

4.2.2 Potential mechanisms for the inhibitory effect of ciprofloxacin on AMP induction

By promoting acetylation of histones, butyrate or its analogue might facilitate transcription of the *CAMP* gene [220, 221, 223]. Induced phosphorylation of histone H3 during interphase have been shown to activate several genes such as c-fos, c-jun, additional AP-1 family genes and the oncogene c-myc [262]. Moreover, association between phosphorylation and acetylation of H3, especially between phosphorylation at Ser10 and acetylation at Lys14, has been implicated for transcriptional activation [263]. Therefore, we analyzed acetylation of histone H3 and H4 and phosphorylation of H3 to investigate the epigenetic modifications, potentially responsible for the effect of NaB and/or ciprofloxacin (or pivmecillinam) on the expression of the *CAMP* gene and HBD transcripts in HT-29 cells. Western blot analysis of histone extract revealed enhanced phosphorylation of H3 at Ser10 and acetylation of H3 and H4 at Lys14 and Lys 16, respectively, within 2 hour of NaB stimulation that persisted up to 24 hours. Interestingly, ciprofloxacin inhibited induced phosphorylation of H3 in a concentration dependent manner at any time point investigated (2, 4, 6 and 24 hours). However, no effect of ciprofloxacin was observed on induced acetylation of both H3 and H4. These findings indicated certain epigenetic modifications of histones being involved in the induction of AMPs by butyrate and subsequent suppression by ciprofloxacin. In a previous study, butyrate was shown to augment LPS-mediated expression of the gene encoding cyclooxygenase-2 (COX-2) in macrophages, but butyrate alone had no effect [264]. In fact, in conjunction with LPS, butyrate could stimulate both phosphorylation and acetylation of H3 at Ser10 and Lys14, respectively, at the promoter of the COX-2 gene. However, butyrate alone induced phosphorylation but not acetylation of H3 at the

COX-2 promoter, although global increase of acetylation was observed by Western blot analysis [264]. On the other hand, we have proposed that global modification of histone acetylation by PB might facilitate other genes, encoding factors that induce the *CAMP* gene [223]. Determination of gene-specific modifications of histones or other gene-encoded factors would better elucidate the mechanisms behind our findings. When cells were stimulated only with ciprofloxacin, no changes in histone phosphorylation and acetylation was observed. Pivmecillinam alone or in conjunction with NaB displayed no apparent effect on histone modifications.

MAPK signaling pathways have been shown to be involved in butyrate induction of the *CAMP* gene [217, 219, 221] as well as histone phosphorylation [263]. However, we did not observe any change in the expression of phosphorylated ERK 1/2 or p-38 MAPK at any time point after stimulation (5 minutes to 24 hours) with butyrate and/or ciprofloxacin (or pivmecillinam).

4.2.3 Effects of butyrate and combination of butyrate and ciprofloxacin on the expression of additional genes in colonic epithelial cells

Microarray analysis of extracted RNA from HT-29 cells was performed in order to find out the effects of butyrate alone or in combination with ciprofloxacin on a broad spectrum of genes. Numerous genes followed the similar pattern of expression as *CAMP* gene and gene encoding HBD-3, while some others followed the opposite pattern. Genes that were co-expressed with the *CAMP* gene or HBD-3 transcript included those encoding G-coupled protein receptors (GPCRs), kallikrein 1, S100 calcium binding proteins and RNase A. GPCRs have been reported to mediate the function of SCFA including butyrate [265, 266] and might also regulate AMP expression in colonic epithelial cells upon stimulation with butyrate and/or ciprofloxacin. By modulating the expression of kallikrein family members, butyrate and also ciprofloxacin could regulate the processing and activity of LL-37 [118]. In agreement with our findings, doxycycline has very recently been reported to inhibit the generation of active LL-37 by suppressing kallikrein-related peptidases [267]. Members of the S100 calcium binding proteins and RNase superfamily exhibit antimicrobial and immunomodulatory properties and hence contribute to the host defense [268, 269].

4.2.4 Implication of antibiotic-mediated suppression of AMPs on *Clostridium difficile* overgrowth in the gut

Altered balance of normal gut flora after antibiotic treatment is regarded as the classical cause of CDAD development. Impaired immune responses have also been reported as important risk factors for CDAD [64]. To investigate if the suppression of AMPs in gut epithelia by antibiotics is associated with CDAD, antibacterial activity of LL-37 against *C. difficile* was evaluated *in vitro.* Incubation of two clinical isolates of ciprofloxacin-resistant *C. difficile* with 5 μM LL-37 resulted in two log decrease of the colony forming units (CFU). In contrast two log increase of CFU was observed in media without LL-37. In a previous study, α- defensins including HD-5 were shown to inhibit the cytotoxic effect of *C. difficile* toxin B [270]. Therefore, reduction of the expression of LL-37, HBD-3 or additional AMPs after antibiotic treatment may allow overgrowth

of *C. difficile* in the gut and subsequent toxin-mediated damage. This hypothesis is supported by a previous finding, where downregulation of intestinal expression of RegIIIc, a secreted C-type lectin in antibiotic- treated mice was shown to increase the colonization of gut by vancomycin-resistant *Enterococcus* [271]. Furthermore, role of certain AMPs in regulating the composition of the gut flora has been reported [90]. Hence, Inhibition of the gut AMP expression can also contribute to the imbalance of microbial niche, promoting CDAD.

5 CONCLUSIONS

- Downregulation of cathelicidin CAP-18 in the large intestinal epithelia of a rabbit model of shigellosis confirms our previous findings in patients with shigellosis (Figure 4). CAP-18 is also downregulated in the epithelia of lung and trachea of infected rabbits (paper I and III).

- Oral treatment of *Shigella*-infected rabbits with NaB or PB counteracts the downregulation of CAP-18 in large intestinal epithelia and promotes secretion and processing of CAP-18 proform into active mature peptide in gut lumen. These activities correlate with reduced bacterial load and improved clinical and pathological outcome of shigellosis (Figure 4) (paper I and III).

- Use of NaB enema as adjunct therapy to antibiotics in patients with shigellosis, leads to earlier reduction of inflammation and healing of mucosal damage of rectum along with enhanced expression and prolonged secretion of LL-37 in rectal epithelia and lumen, respectively (Figure 4) (paper II).

- Restoration of downregulated CAP-18 in respiratory epithelia of *Shigella*-infected rabbits by oral treatment with NaB or PB may lead to protection from secondary respiratory infections during shigellosis (paper III).

- Suppression of AMPs and other innate immune components by antibiotics in colonic epithelia may contribute to CDAD, which has traditionally been known to occur via antibiotic-mediated imbalance of the intestinal flora (Figure 5) (paper IV).

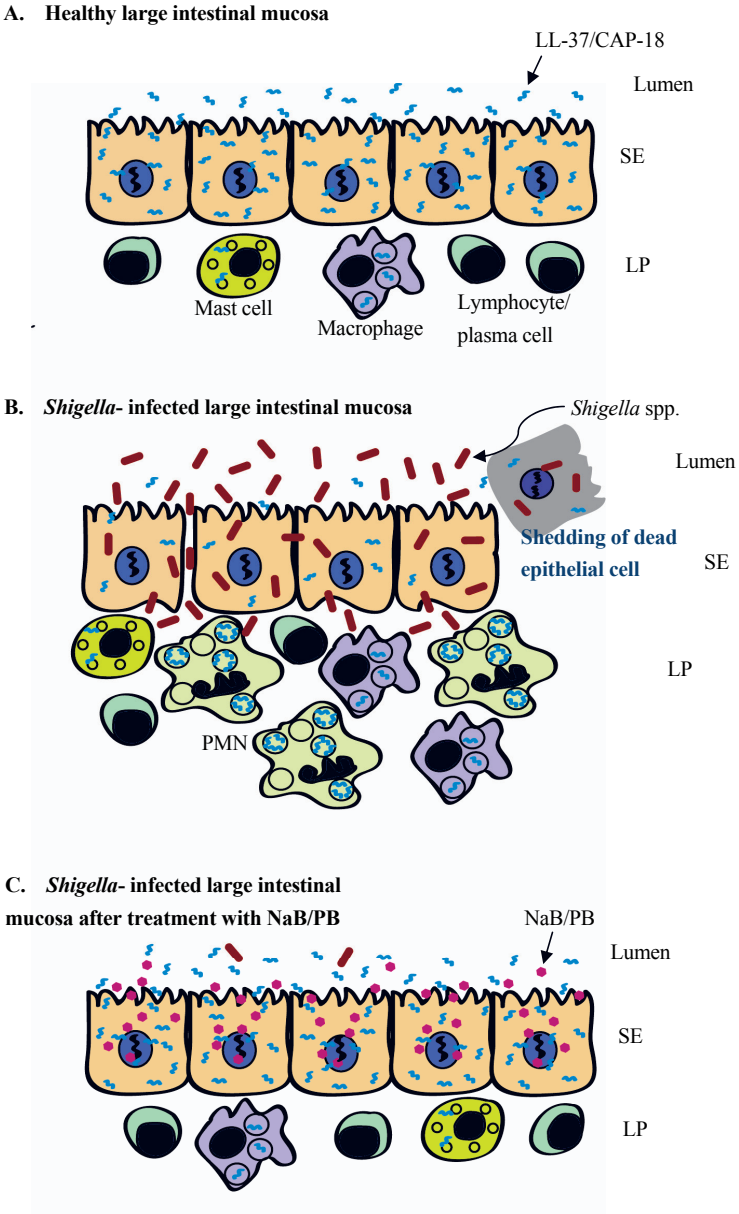
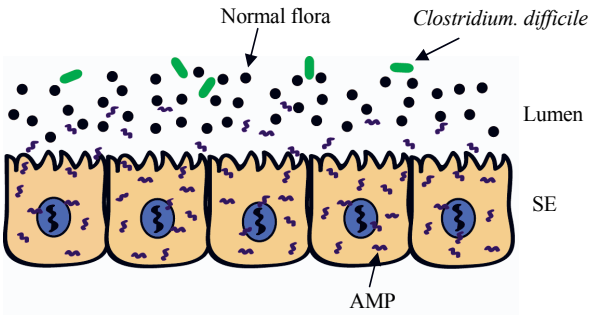


Figure 4: Counteraction of *Shigella*-mediated downregulation of cathelicidins in large intestinal epithelia after treatment with NaB or PB, a schematic representation. (A) Abundant expression of LL-37/CAP-18 in large intestinal epithelia and also in the lumen of healthy host. (B) Downregulation of epithelial expression of LL-37/CAP-18 and influx of LL-37/CAP-18 producing inflammatory cells in the LP during *Shigella* infection. Detachment of dead epithelial cell from the epithelial lining indicates epithelial erosion. (C) After treatment with NaB or PB, LL-37/CAP-18 expression in the epithelia is restored and bacterial load is reduced, while inflammatory cells disappear from the LP. SE: surface epithelia; LP: lamina propria; NaB: sodium butyrate; PB: sodium-4-phenylbutyrate.

A. Large intestinal mucosa and lumen in normal condition



B. Large intestinal mucosa and lumen after antibiotic treatment

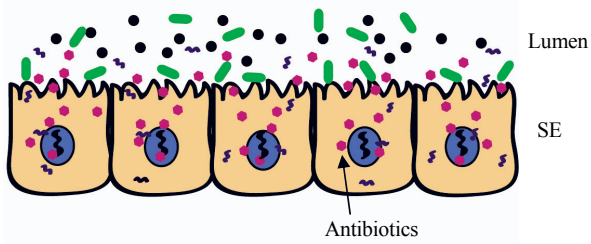


Figure 5: A schematic representation of colonization of large intestinal epithelia by *Clostridium difficile* after antibiotic-mediated disruption of the normal gut flora and suppression of AMPs. (A) Normal gut flora and AMPs create a preventive barrier against *C. difficile*. (B) Antibiotics disturb the balance of the normal flora as well as suppress the epithelial expression of AMPs, allowing overgrowth of *C.difficile* and subsequent colonization of the epithelia. AMP: antimicrobial peptide; SE: surface epithelia; LP: lamina propria.

6 FUTURE PERSPECTIVE

Our findings indicate that the induction of AMPs at the site of infection by administration of extrinsic compounds can be a potential strategy of alternative or adjunct therapy to commonly used antibiotics. However, at present, induction studies have been limited to very few AMPs i.e. cathelicidins and defensins. Detailed understanding of the stimulation profile of additional antimicrobial peptides/proteins will put forward this therapeutic strategy. In addition, novel inducing components need to be explored that would upregulate numerous AMPs at infection sites. In order to continue the search for novel inducers, it is important to develop large scale screening methods. In this perspective, a colonic epithelial cell line, expressing luciferase under *CAMP* regulatory elements has recently been constructed in our laboratory. This stable cell line has responded successfully to known inducers and several novel inducers have been demonstrated for LL-37 expression (unpublished data). Cytotoxic and proinflammatory effects of excessive AMPs and the inducers per se should also be taken into account in order to make this strategy feasible.

Regarding infections of the gut, the role of the mucus layer is a fascinating area of research. The mucus layer, overlying the epithelial surface of the gut, constitutes a physical barrier against pathogens and also against commensals, preventing direct contact with the epithelia. It also provides a chemical barrier by harboring effector molecules such as secretory IgA and several antimicrobial peptides/proteins, secreted from the underlying mucosa [272, 273]. It will be interesting to know, the expression profile of AMPs in the mucus layer in shigellosis, in enteric infections in general and after treatment with inducing components. The effect of antibiotics on mucosal expression of AMPs will also be intriguing.

7 ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Biochemistry and Biophysics (MBB), Karolinska Institutet, Stockholm, Sweden in collaboration with International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b), Dhaka, Bangladesh. I am indebted to many peoples throughout this journey.

I acknowledge **the patients** who generously agreed to participate in the clinical trial.

Birgitta Agerberth, my main supervisor: I am grateful to you for accepting me as a PhD student and for your excellent scientific guidance. Many thanks for your always encouraging words and giving me the freedom to explore my own ideas. Your door has always been open to discuss scientific and also personal matters. I remember, how concern you were about me, when I first came to Sweden leaving my family at home.

Rubhana Raqib, my co-supervisor: Heartfelt gratitude to you for introducing me to the world of science. You accepted me as a Master's thesis student almost 14 years back and since then guiding me to build up my scientific career. During this long journey, you have always kept faith on me and arranged this collaborative PhD program.

Jan Johansson, my mentor: I am indebted to you for being my mentor.

Hans Jörnvall: Thank you for allowing me to work in the Division of Chemistry I. I appreciate your critical questions and insightful advice during 'Thursday Seminar'. During 'Monday Meeting', listening to your experiences about birds, nature and peoples from different parts of the world was wonderful.

Jesper Z. Haeggström: Thank you for letting me continue my study in the Division of Chemistry II. Thanks also for sharing your extensive knowledge about lipid mediators in 'Division Meeting' and 'Chemical Biology Course'.

Alejandro Cravioto, Executive Director of icddr, b: I express my sincere gratitude to you for kindly giving your consent for this collaborative PhD program.

Firdausi Qadri: I convey my sincere thanks to you for permitting me to work as a Master's thesis student in the Immunology Laboratory of icddr, b.

Gudmundur H. Gudmundsson: I sincerely appreciate your constructive and critical suggestions in project planning and manuscript writing.

Jan Andersson: I convey my acknowledgement to you for your valuable feedback in the projects and manuscripts.

Peter Bergman: Thanks for your fascinating input in designing experiments and manuscript writing. It has always been fantastic to discuss with you about science and to hear from you about new exciting findings.

Thanks to all **co-authors** in different manuscript for your valuable contribution.

I acknowledge the enormous contribution of **Emon, Mily, Snigdha, Mamun** and **Gul Ara** in performing the animal experimentation and extensive lab works. Thanks **Rekha** for helping me in lab works, statistical analyses and graph making. Thanks **Dr. Arif** and **Dr. Fahima** for carefully monitoring the patients and collecting clinical data. Special thanks to **Dr. Nur Haque Alam** for performing the sigmoidoscopy of the patients and collecting biopsy specimens. Thanks to **all individuals,** who helped in randomization, blinding, concealing and implementation of interventions and taking care of patients. Thanks to **Asit, Amirul** and **Saidul** for collecting and extracting stool specimens and helping with storage of samples. Thanks to **KM Nasirul Islam** and **other staffs** of the animal resource facilities of icddr, b for helping us with the animal experimentation.

Thanks to **Roger Strömberg** for GC-MS analyses to detect butyrate levels in serum. Thanks to **Shah Jalal** for providing the clinical isolates of *Clostridium difficile* and facilities to work with these bugs. Thanks **Kristmundur Sigmundsson** for your help in operating Operetta Image analyzer.

Thanks to my colleagues in the AMP lab, **Monica, Ylva, Andreas, Erica** and **Frank** for providing a pleasant atmosphere and for fruitful technical and scientific discussion. Thanks **Monica** to keep the lab well-organized, clean and tidy and for ordering stuffs. Thanks **Andreas** for fixing technical and computer related problems. Good luck with your thesis.

Thanks **Jia, Min** and **Dolores** for sharing reagents and for being good friends. I would like to extend my warmest thanks to all my colleagues in the division of chemistry I and II. Thanks **Essam, Michael and Linus** for making fun every now and then. Thanks to **Ingegard Nylander** and **Victoria Balabanova** for all the secretarial help.

Many thanks to **Lennert Lindbom, Susanna Brighenti, Ellinor** and **Anne** for enthusiastic discussion in 'SSF meeting'.

Thanks to my present and former colleagues at icddr, b for your generous support. Special thanks to **Firoz Ahmed** for introducing me to Rubhana apa.

I acknowledge the influence of my **teachers, relatives** and **many other peoples** on my upbringing. Thanks to all **my friends** whom I always share my joy with and who have always been beside me during the bad phases of my life.

Warmest gratitude to **Mukti apa** and **Apu vai, Najnin** and **Afsar vai, Mousumi** and **Jubayer, Shapla** and **Saiham, Rekha** and **Emon, Mosfique vai** and **Emily vabi,** and **Sayem**. With your company, we never felt like staying away from Bangladesh. It was nice to see the kids playing, fighting and screaming to-gether. Thanks **Mridul da** and **Tapasi didi**, **Deepak** and **Suvarna, Raj** and **Jolly** for being good family friends.

Thanks to **Daniel, Karim vai, Rana vai, Robi vai, Murad vai, Sanjib** and **Mamun** for the nice time in Jaggargatan. Thanks **Ranjan** and **Sandhi** for being good friends.

Thanks **Ahsan** for making our Paris tour possible. We really had a wonderful time in Paris. Thanks **Sanjib** for arranging the trip to Switzerland. Without you, we would miss the outstanding experience of our life. Thanks **Robi vai** for the fantastic boat-trip to Helsinki.

Hannan mama and **Rumi mami**: You were like our local guardians. Thanks for your care and support.

My mother, your never-ending love and mental strength have encouraged me coming this far. **My father**, we have grown up seeing your honesty and dedication to work. **My brother and his family**, many thanks for staying with parents, which made it easy for me to stay abroad for such a long time. **My sister and her family**, thanks for your always inspiring words. **My parents-in-law**, I admire your affection and constant support. **My sister-in-law and her family**, thanks for all the lovely addas. We will have more fun in the future.

My wife, Susmita, I would never reach where I am now without your love, understanding and great sacrifice. **Soham, my son** and **Ridhima, my daughter,** two precious gifts of God. Seeing your smiling faces, takes away all the after-work tiredness and frustration of life. You are the real meaning of life.

My salary was supported by **'the Karolinska Institutet faculty Fund (KID grant)'**. The research preparatory course was supported by **'the Svenska Institutet'**. The research was financially supported by **'the Swedish Agency for Research Cooperation with Developing Countries (Sida/SAREC)'**, **'the Swedish Research Council'**, **'the Swedish Strategic foundation (SSF)'**, **'Karolinska Institutet'** and **'icddr, b'**.

8 REFERENCES

1. Bardhan P, Faruque AS, Naheed A, Sack DA: **Decrease in shigellosis-related deaths without Shigella spp.-specific interventions, Asia**. *Emerg Infect Dis* 2010, **16**(11):1718-1723.
2. Butler T, Dunn D, Dahms B, Islam M: **Causes of death and the histopathologic findings in fatal shigellosis**. *Pediatr Infect Dis J* 1989, **8**(11):767-772.
3. Dutta P, Mitra U, Rasaily R, Bhattacharya SK, Bhattacharya MK, Manna B, Gupta A, Kundu B: **Assessing the cause of in-patients pediatric diarrheal deaths: an analysis of hospital records**. *Indian Pediatr* 1995, **32**(3):313-321.
4. Mitra AK, Engleberg NC, Glass RI, Chowdhury MK: **Fatal dysentery in rural Bangladesh**. *J Diarrhoeal Dis Res* 1990, **8**(1-2):12-17.
5. Bennish ML, Salam MA, Wahed MA: **Enteric protein loss during shigellosis**. *Am J Gastroenterol* 1993, **88**(1):53-57.
6. Black RE, Brown KH, Becker S: **Effects of diarrhea associated with specific enteropathogens on the growth of children in rural Bangladesh**. *Pediatrics* 1984, **73**(6):799-805.
7. Hannu T, Mattila L, Siitonen A, Leirisalo-Repo M: **Reactive arthritis attributable to Shigella infection: a clinical and epidemiological nationwide study**. *Ann Rheum Dis* 2005, **64**(4):594-598.
8. Wang LH, Fang XC, Pan GZ: **Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis**. *Gut* 2004, **53**(8):1096-1101.
9. Mounier J, Vasselon T, Hellio R, Lesourd M, Sansonetti PJ: **Shigella flexneri enters human colonic Caco-2 epithelial cells through the basolateral pole**. *Infect Immun* 1992, **60**(1):237-248.
10. Perdomo OJ, Cavaillon JM, Huerre M, Ohayon H, Gounon P, Sansonetti PJ: **Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis**. *J Exp Med* 1994, **180**(4):1307-1319.
11. Sansonetti PJ, Arondel J, Cantey JR, Prevost MC, Huerre M: **Infection of rabbit Peyer's patches by Shigella flexneri: effect of adhesive or invasive bacterial phenotypes on follicle-associated epithelium**. *Infect Immun* 1996, **64**(7):2752-2764.
12. Schroeder GN, Hilbi H: **Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion**. *Clin Microbiol Rev* 2008, **21**(1):134-156.
13. Sasakawa C: **A new paradigm of bacteria-gut interplay brought through the study of Shigella**. *Proc Jpn Acad Ser B Phys Biol Sci* 2010, **86**(3):229-243.
14. Zychlinsky A, Prevost MC, Sansonetti PJ: **Shigella flexneri induces apoptosis in infected macrophages**. *Nature* 1992, **358**(6382):167-169.
15. Sansonetti PJ, Phalipon A, Arondel J, Thirumalai K, Banerjee S, Akira S, Takeda K, Zychlinsky A: **Caspase-1 activation of IL-1beta and IL-18 are essential for Shigella flexneri-induced inflammation**. *Immunity* 2000, **12**(5):581-590.
16. Zychlinsky A, Fitting C, Cavaillon JM, Sansonetti PJ: **Interleukin 1 is released by murine macrophages during apoptosis induced by Shigella flexneri**. *J Clin Invest* 1994, **94**(3):1328-1332.
17. Sansonetti PJ, Arondel J, Cavaillon JM, Huerre M: **Role of interleukin-1 in the pathogenesis of experimental shigellosis**. *J Clin Invest* 1995, **96**(2):884-892.
18. Sansonetti PJ: **Microbes and microbial toxins: paradigms for microbial-mucosal interactions III. Shigellosis: from symptoms to molecular pathogenesis**. *Am J Physiol Gastrointest Liver Physiol* 2001, **280**(3):G319-323.
19. Pedron T, Thibault C, Sansonetti PJ: **The invasive phenotype of Shigella flexneri directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2**. *J Biol Chem* 2003, **278**(36):33878-33886.
20. Philpott DJ, Yamaoka S, Israel A, Sansonetti PJ: **Invasive Shigella flexneri activates NF-kappa B through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells**. *J Immunol* 2000, **165**(2):903-914.

21. Sansonetti PJ, Arondel J, Huerre M, Harada A, Matsushima K: **Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis**. *Infect Immun* 1999, **67**(3):1471-1480.
22. Islam D, Veress B, Bardhan PK, Lindberg AA, Christensson B: **In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis**. *Infect Immun* 1997, **65**(2):739-749.
23. Raqib R, Lindberg AA, Wretlind B, Bardhan PK, Andersson U, Andersson J: **Persistence of local cytokine production in shigellosis in acute and convalescent stages**. *Infect Immun* 1995, **63**(1):289-296.
24. Raqib R, Mia SM, Qadri F, Alam TI, Alam NH, Chowdhury AK, Mathan MM, Andersson J: **Innate immune responses in children and adults with Shigellosis**. *Infect Immun* 2000, **68**(6):3620-3629.
25. Raqib R, Moly PK, Sarker P, Qadri F, Alam NH, Mathan M, Andersson J: **Persistence of mucosal mast cells and eosinophils in Shigella-infected children**. *Infect Immun* 2003, **71**(5):2684-2692.
26. Raqib R, Reinholt FP, Bardhan PK, Karnell A, Lindberg AA: **Immunopathological patterns in the rectal mucosa of patients with shigellosis: expression of HLA-DR antigens and T-lymphocyte subsets**. *APMIS* 1994, **102**(5):371-380.
27. Raqib R, Ekberg C, Sharkar P, Bardhan PK, Zychlinsky A, Sansonetti PJ, Andersson J: **Apoptosis in acute shigellosis is associated with increased production of Fas/Fas ligand, perforin, caspase-1, and caspase-3 but reduced production of Bcl-2 and interleukin-2**. *Infect Immun* 2002, **70**(6):3199-3207.
28. Mathan MM, Mathan VI: **Ultrastructural pathology of the rectal mucosa in Shigella dysentery**. *Am J Pathol* 1986, **123**(1):25-38.
29. Gorden J, Small PL: **Acid resistance in enteric bacteria**. *Infect Immun* 1993, **61**(1):364-367.
30. Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, Gudmundsson G: **Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator**. *Nat Med* 2001, **7**(2):180-185.
31. Sperandio B, Regnault B, Guo J, Zhang Z, Stanley SL, Jr., Sansonetti PJ, Pedron T: **Virulent Shigella flexneri subverts the host innate immune response through manipulation of antimicrobial peptide gene expression**. *J Exp Med* 2008, **205**(5):1121-1132.
32. Deretic V, Levine B: **Autophagy, immunity, and microbial adaptations**. *Cell Host Microbe* 2009, **5**(6):527-549.
33. Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C: **Escape of intracellular Shigella from autophagy**. *Science* 2005, **307**(5710):727-731.
34. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: **Neutrophil extracellular traps kill bacteria**. *Science* 2004, **303**(5663):1532-1535.
35. Ashida H, Ogawa M, Kim M, Suzuki S, Sanada T, Punginelli C, Mimuro H, Sasakawa C: **Shigella deploy multiple countermeasures against host innate immune responses**. *Curr Opin Microbiol* 2011, **14**(1):16-23.
36. Shtrichman R, Samuel CE: **The role of gamma interferon in antimicrobial immunity**. *Curr Opin Microbiol* 2001, **4**(3):251-259.
37. Way SS, Borczuk AC, Dominitz R, Goldberg MB: **An essential role for gamma interferon in innate resistance to Shigella flexneri infection**. *Infect Immun* 1998, **66**(4):1342-1348.
38. Raqib R, Ljungdahl A, Lindberg AA, Andersson U, Andersson J: **Local entrapment of interferon gamma in the recovery from Shigella dysenteriae type 1 infection**. *Gut* 1996, **38**(3):328-336.
39. Raqib R, Lindberg AA, Bjork L, Bardhan PK, Wretlind B, Andersson U, Andersson J: **Down-regulation of gamma interferon, tumor necrosis factor type I, interleukin 1 (IL-1) type I, IL-3, IL-4, and transforming growth factor beta type I receptors at the local site during the acute phase of Shigella infection**. *Infect Immun* 1995, **63**(8):3079-3087.

40. Raqib R, Gustafsson A, Andersson J, Bakhiet M: **A systemic downregulation of gamma interferon production is associated with acute shigellosis**. *Infect Immun* 1997, **65**(12):5338-5341.
41. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB: **Clinical trials of Shigella vaccines: two steps forward and one step back on a long, hard road**. *Nat Rev Microbiol* 2007, **5**(7):540-553.
42. Raqib R, Qadri F, SarkEr P, Mia SM, Sansonnetti PJ, Albert MJ, Andersson J: **Delayed and reduced adaptive humoral immune responses in children with shigellosis compared with in adults**. *Scand J Immunol* 2002, **55**(4):414-423.
43. Oberhelman RA, Kopecko DJ, Salazar-Lindo E, Gotuzzo E, Buysse JM, Venkatesan MM, Yi A, Fernandez-Prada C, Guzman M, Leon-Barua R *et al*: **Prospective study of systemic and mucosal immune responses in dysenteric patients to specific Shigella invasion plasmid antigens and lipopolysaccharides**. *Infect Immun* 1991, **59**(7):2341-2350.
44. Cohen D, Green MS, Block C, Slepon R, Ofek I: **Prospective study of the association between serum antibodies to lipopolysaccharide O antigen and the attack rate of shigellosis**. *J Clin Microbiol* 1991, **29**(2):386-389.
45. Phalipon A, Kaufmann M, Michetti P, Cavaillon JM, Huerre M, Sansonetti P, Kraehenbuhl JP: **Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of Shigella flexneri lipopolysaccharide protects against murine experimental shigellosis**. *J Exp Med* 1995, **182**(3):769-778.
46. Van de Verg LL, Herrington DA, Boslego J, Lindberg AA, Levine MM: **Age-specific prevalence of serum antibodies to the invasion plasmid and lipopolysaccharide antigens of Shigella species in Chilean and North American populations**. *J Infect Dis* 1992, **166**(1):158-161.
47. Islam D, Christensson B: **Disease-dependent changes in T-cell populations in patients with shigellosis**. *APMIS* 2000, **108**(4):251-260.
48. Jehl SP, Doling AM, Giddings KS, Phalipon A, Sansonetti PJ, Goldberg MB, Starnbach MN: **Antigen-specific CD8(+) T cells fail to respond to Shigella flexneri**. *Infect Immun* 2011, **79**(5):2021-2030.
49. Konradt C, Frigimelica E, Nothelfer K, Puhar A, Salgado-Pabon W, di Bartolo V, Scott-Algara D, Rodrigues CD, Sansonetti PJ, Phalipon A: **The Shigella flexneri type three secretion system effector IpgD inhibits T cell migration by manipulating host phosphoinositide metabolism**. *Cell Host Microbe* 2011, **9**(4):263-272.
50. Sellge G, Magalhaes JG, Konradt C, Fritz JH, Salgado-Pabon W, Eberl G, Bandeira A, Di Santo JP, Sansonetti PJ, Phalipon A: **Th17 cells are the dominant T cell subtype primed by Shigella flexneri mediating protective immunity**. *J Immunol* 2010, **184**(4):2076-2085.
51. Phalipon A, Sansonetti PJ: **Shigella's ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival?** *Immunol Cell Biol* 2007, **85**(2):119-129.
52. Chen Y, Smith MR, Thirumalai K, Zychlinsky A: **A bacterial invasin induces macrophage apoptosis by binding directly to ICE**. *EMBO J* 1996, **15**(15):3853-3860.
53. Ogawa M, Handa Y, Ashida H, Suzuki M, Sasakawa C: **The versatility of Shigella effectors**. *Nat Rev Microbiol* 2008, **6**(1):11-16.
54. World Health Organization: **Guidelines for the control of shigellosis, including epidemics due to Shigella dysenteriae type 1**. Geneva: WHO Press 2005: 1-64.
55. Bhattacharya D, Purushottaman SA, Bhattacharjee H, Thamizhmani R, Sudharama SD, Manimunda SP, Bharadwaj AP, Singhania M, Roy S: **Rapid emergence of third-generation cephalosporin resistance in Shigella sp. isolated in Andaman and Nicobar Islands, India**. *Microb Drug Resist* 2011, **17**(2):329-332.
56. Urvashi, Saxena S, Dutta R: **Antimicrobial resistance pattern of Shigella species over five years at a tertiary-care teaching hospital in north India**. *J Health Popul Nutr* 2011, **29**(3):292-295.
57. Venkatesan MM, Ranallo RT: **Live-attenuated Shigella vaccines**. *Expert Rev Vaccines* 2006, **5**(5):669-686.

58. Coster TS, Hoge CW, VanDeVerg LL, Hartman AB, Oaks EV, Venkatesan MM, Cohen D, Robin G, Fontaine-Thompson A, Sansonetti PJ *et al*: **Vaccination against shigellosis with attenuated Shigella flexneri 2a strain SC602**. *Infect Immun* 1999, **67**(7):3437-3443.
59. Rahman KM, Arifeen SE, Zaman K, Rahman M, Raqib R, Yunus M, Begum N, Islam MS, Sohel BM, Venkatesan M *et al*: **Safety, dose, immunogenicity, and transmissibility of an oral live attenuated Shigella flexneri 2a vaccine candidate (SC602) among healthy adults and school children in Matlab, Bangladesh**. *Vaccine* 2011, **29**(6):1347-1354.
60. Launay O, Sadorge C, Jolly N, Poirier B, Bechet S, van der Vliet D, Seffer V, Fenner N, Dowling K, Giemza R *et al*: **Safety and immunogenicity of SC599, an oral live attenuated Shigella dysenteriae type-1 vaccine in healthy volunteers: results of a Phase 2, randomized, double-blind placebo-controlled trial**. *Vaccine* 2009, **27**(8):1184-1191.
61. Sadorge C, Ndiaye A, Beveridge N, Frazer S, Giemza R, Jolly N, Johnson J, Liddy H, Cosgrove CA, Allavena P *et al*: **Phase 1 clinical trial of live attenuated Shigella dysenteriae type-1 DeltaicsA Deltaent Deltafep DeltastxA:HgR oral vaccine SC599 in healthy human adult volunteers**. *Vaccine* 2008, **26**(7):978-987.
62. Kaminski RW, Oaks EV: **Inactivated and subunit vaccines to prevent shigellosis**. *Expert Rev Vaccines* 2009, **8**(12):1693-1704.
63. Hogenauer C, Hammer HF, Krejs GJ, Reisinger EC: **Mechanisms and management of antibiotic-associated diarrhea**. *Clin Infect Dis* 1998, **27**(4):702-710.
64. Vaishnavi C: **Established and potential risk factors for Clostridum difficile infection**. *Indian J Med Microbiol* 2009, **27**(4):289-300.
65. Deneve C, Janoir C, Poilane I, Fantinato C, Collignon A: **New trends in Clostridium difficile virulence and pathogenesis**. *Int J Antimicrob Agents* 2009, **33 Suppl 1**:S24-28.
66. Voth DE, Ballard JD: **Clostridium difficile toxins: mechanism of action and role in disease**. *Clin Microbiol Rev* 2005, **18**(2):247-263.
67. Pothoulakis C: **Effects of Clostridium difficile toxins on epithelial cell barrier**. *Ann N Y Acad Sci* 2000, **915**:347-356.
68. Riegler M, Sedivy R, Pothoulakis C, Hamilton G, Zacherl J, Bischof G, Cosentini E, Feil W, Schiessel R, LaMont JT *et al*: **Clostridium difficile toxin B is more potent than toxin A in damaging human colonic epithelium in vitro**. *J Clin Invest* 1995, **95**(5):2004-2011.
69. Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S *et al*: **Toxin B is essential for virulence of Clostridium difficile**. *Nature* 2009, **458**(7242):1176-1179.
70. Drudy D, Fanning S, Kyne L: **Toxin A-negative, toxin B-positive Clostridium difficile**. *Int J Infect Dis* 2007, **11**(1):5-10.
71. Barbut F, Decre D, Lalande V, Burghoffer B, Noussair L, Gigandon A, Espinasse F, Raskine L, Robert J, Mangeol A *et al*: **Clinical features of Clostridium difficile-associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains**. *J Med Microbiol* 2005, **54**(Pt 2):181-185.
72. Vaishnavi C: **Clostridium difficile infection: clinical spectrum and approach to management**. *Indian J Gastroenterol* 2011, **30**(6):245-254.
73. Johnson S: **Recurrent Clostridium difficile infection: causality and therapeutic approaches**. *Int J Antimicrob Agents* 2009, **33 Suppl 1**:S33-36.
74. Kee VR: **Clostridium difficile infection in older adults: a review and update on its management**. *Am J Geriatr Pharmacother* 2012, **10**(1):14-24.
75. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH: **Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA)**. *Infect Control Hosp Epidemiol* 2010, **31**(5):431-455.
76. Lowy I, Molrine DC, Leav BA, Blair BM, Baxter R, Gerding DN, Nichol G, Thomas WD, Jr., Leney M, Sloan S *et al*: **Treatment with monoclonal**

antibodies against Clostridium difficile toxins. *N Engl J Med* 2010, **362**(3):197-205.
77. Zasloff M: **Antimicrobial peptides of multicellular organisms**. *Nature* 2002, **415**(6870):389-395.
78. Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG: **Sequence and specificity of two antibacterial proteins involved in insect immunity**. *Nature* 1981, **292**(5820):246-248.
79. Selsted ME, Brown DM, DeLange RJ, Lehrer RI: **Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages**. *J Biol Chem* 1983, **258**(23):14485-14489.
80. Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI: **Primary structures of three human neutrophil defensins**. *J Clin Invest* 1985, **76**(4):1436-1439.
81. Zasloff M: **Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor**. *Proc Natl Acad Sci U S A* 1987, **84**(15):5449-5453.
82. Wang G, Li X, Wang Z: **APD2: the updated antimicrobial peptide database and its application in peptide design**. *Nucleic Acids Res* 2009, **37**(Database issue):D933-937.
83. Giuliani A, Rinaldi AC: **Beyond natural antimicrobial peptides: multimeric peptides and other peptidomimetic approaches**. *Cell Mol Life Sci* 2011, **68**(13):2255-2266.
84. Brogden KA: **Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?** *Nat Rev Microbiol* 2005, **3**(3):238-250.
85. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B: **Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37**. *J Biol Chem* 1998, **273**(6):3718-3724.
86. Yang D, Chertov O, Oppenheim JJ: **The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity**. *Cell Mol Life Sci* 2001, **58**(7):978-989.
87. Zanetti M: **Cathelicidins, multifunctional peptides of the innate immunity**. *J Leukoc Biol* 2004, **75**(1):39-48.
88. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC: **Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein**. *Infect Immun* 1995, **63**(4):1291-1297.
89. Larrick JW, Morgan JG, Palings I, Hirata M, Yen MH: **Complementary DNA sequence of rabbit CAP18--a unique lipopolysaccharide binding protein**. *Biochem Biophys Res Commun* 1991, **179**(1):170-175.
90. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, Teggatz P, Barman M, Hayward M, Eastwood D *et al*: **Enteric defensins are essential regulators of intestinal microbial ecology**. *Nat Immunol* 2010, **11**(1):76-83.
91. Baroni A, Donnarumma G, Paoletti I, Longanesi-Cattani I, Bifulco K, Tufano MA, Carriero MV: **Antimicrobial human beta-defensin-2 stimulates migration, proliferation and tube formation of human umbilical vein endothelial cells**. *Peptides* 2009, **30**(2):267-272.
92. Heilborn JD, Nilsson MF, Kratz G, Weber G, Sorensen O, Borregaard N, Stahle-Backdahl M: **The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium**. *J Invest Dermatol* 2003, **120**(3):379-389.
93. Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, Issbrucker K, Unterberger P, Zaiou M, Lebherz C *et al*: **An angiogenic role for the human peptide antibiotic LL-37/hCAP-18**. *J Clin Invest* 2003, **111**(11):1665-1672.
94. Otte JM, Zdebik AE, Brand S, Chromik AM, Strauss S, Schmitz F, Steinstraesser L, Schmidt WE: **Effects of the cathelicidin LL-37 on intestinal epithelial barrier integrity**. *Regul Pept* 2009, **156**(1-3):104-117.
95. Shaykhiev R, Beisswenger C, Kandler K, Senske J, Puchner A, Damm T, Behr J, Bals R: **Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure**. *Am J Physiol Lung Cell Mol Physiol* 2005, **289**(5):L842-848.

96. Maier VH, Dorn KV, Gudmundsdottir BK, Gudmundsson GH: **Characterisation of cathelicidin gene family members in divergent fish species**. *Mol Immunol* 2008, **45**(14):3723-3730.
97. Lynn DJ, Higgs R, Gaines S, Tierney J, James T, Lloyd AT, Fares MA, Mulcahy G, O'Farrelly C: **Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken**. *Immunogenetics* 2004, **56**(3):170-177.
98. Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, Wang A, Lin D, Lai R: **Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics**. *PLoS One* 2008, **3**(9):e3217.
99. Zanetti M, Gennaro R, Romeo D: **Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain**. *FEBS Lett* 1995, **374**(1):1-5.
100. Zaiou M, Nizet V, Gallo RL: **Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence**. *J Invest Dermatol* 2003, **120**(5):810-816.
101. Agerberth B, Gunne H, Odeberg J, Kogner P, Boman HG, Gudmundsson GH: **FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis**. *Proc Natl Acad Sci U S A* 1995, **92**(1):195-199.
102. Gudmundsson GH, Agerberth B, Odeberg J, Bergman T, Olsson B, Salcedo R: **The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes**. *Eur J Biochem* 1996, **238**(2):325-332.
103. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH: **The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations**. *Blood* 2000, **96**(9):3086-3093.
104. Bals R, Wang X, Zasloff M, Wilson JM: **The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface**. *Proc Natl Acad Sci U S A* 1998, **95**(16):9541-9546.
105. Di Nardo A, Vitiello A, Gallo RL: **Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide**. *J Immunol* 2003, **170**(5):2274-2278.
106. Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, Agerberth B, Gudmundsson GH, Gallo RL: **Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus**. *J Invest Dermatol* 2001, **117**(1):91-97.
107. Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, Gudmundsson GH: **The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders**. *J Biol Chem* 1997, **272**(24):15258-15263.
108. Frohm Nilsson M, Sandstedt B, Sorensen O, Weber G, Borregaard N, Stahle-Backdahl M: **The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6**. *Infect Immun* 1999, **67**(5):2561-2566.
109. Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF: **Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium**. *Infect Immun* 2002, **70**(2):953-963.
110. Marchini G, Lindow S, Brismar H, Stabi B, Berggren V, Ulfgren AK, Lonne-Rahm S, Agerberth B, Gudmundsson GH: **The newborn infant is protected by an innate antimicrobial barrier: peptide antibiotics are present in the skin and vernix caseosa**. *Br J Dermatol* 2002, **147**(6):1127-1134.
111. Hase K, Murakami M, Iimura M, Cole SP, Horibe Y, Ohtake T, Obonyo M, Gallo RL, Eckmann L, Kagnoff MF: **Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori**. *Gastroenterology* 2003, **125**(6):1613-1625.

112. Chromek M, Slamova Z, Bergman P, Kovacs L, Podracka L, Ehren I, Hokfelt T, Gudmundsson GH, Gallo RL, Agerberth B *et al*: **The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection**. *Nat Med* 2006, **12**(6):636-641.
113. Murakami M, Dorschner RA, Stern LJ, Lin KH, Gallo RL: **Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk**. *Pediatr Res* 2005, **57**(1):10-15.
114. Murakami M, Ohtake T, Dorschner RA, Gallo RL: **Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva**. *J Dent Res* 2002, **81**(12):845-850.
115. Murakami M, Ohtake T, Dorschner RA, Schittek B, Garbe C, Gallo RL: **Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin**. *J Invest Dermatol* 2002, **119**(5):1090-1095.
116. Gudmundsson GH, Magnusson KP, Chowdhary BP, Johansson M, Andersson L, Boman HG: **Structure of the gene for porcine peptide antibiotic PR-39, a cathelin gene family member: comparative mapping of the locus for the human peptide antibiotic FALL-39**. *Proc Natl Acad Sci U S A* 1995, **92**(15):7085-7089.
117. Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, Borregaard N: **Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3**. *Blood* 2001, **97**(12):3951-3959.
118. Yamasaki K, Schauber J, Coda A, Lin H, Dorschner RA, Schechter NM, Bonnart C, Descargues P, Hovnanian A, Gallo RL: **Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin**. *FASEB J* 2006, **20**(12):2068-2080.
119. Sorensen OE, Gram L, Johnsen AH, Andersson E, Bangsboll S, Tjabringa GS, Hiemstra PS, Malm J, Egesten A, Borregaard N: **Processing of seminal plasma hCAP-18 to ALL-38 by gastricsin: a novel mechanism of generating antimicrobial peptides in vagina**. *J Biol Chem* 2003, **278**(31):28540-28546.
120. Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, Gennaro R: **Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse**. *J Biol Chem* 1997, **272**(20):13088-13093.
121. Termen S, Tollin M, Olsson B, Svenberg T, Agerberth B, Gudmundsson GH: **Phylogeny, processing and expression of the rat cathelicidin rCRAMP: a model for innate antimicrobial peptides**. *Cell Mol Life Sci* 2003, **60**(3):536-549.
122. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL, Leung DY: **Endogenous antimicrobial peptides and skin infections in atopic dermatitis**. *N Engl J Med* 2002, **347**(15):1151-1160.
123. Schauber J, Rieger D, Weiler F, Wehkamp J, Eck M, Fellermann K, Scheppach W, Gallo RL, Stange EF: **Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases**. *Eur J Gastroenterol Hepatol* 2006, **18**(6):615-621.
124. Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, Yamazaki K, Sayama K, Taubman MA, Kurihara H *et al*: **Staphylococcus aureus susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes**. *Infect Immun* 2003, **71**(7):3730-3739.
125. Cobo ER, He C, Hirata K, Hwang G, Tran U, Eckmann L, Gallo RL, Reed SL: **Entamoeba histolytica induces intestinal cathelicidins but is resistant to cathelicidin-mediated killing**. *Infect Immun* 2012, **80**(1):143-149.
126. Bergman P, Johansson L, Wan H, Jones A, Gallo RL, Gudmundsson GH, Hokfelt T, Jonsson AB, Agerberth B: **Induction of the antimicrobial peptide CRAMP in the blood-brain barrier and meninges after meningococcal infection**. *Infect Immun* 2006, **74**(12):6982-6991.
127. Sorensen OE, Cowland JB, Theilgaard-Monch K, Liu L, Ganz T, Borregaard N: **Wound healing and expression of antimicrobial peptides/polypeptides in**

human keratinocytes, a consequence of common growth factors. *J Immunol* 2003, **170**(11):5583-5589.
128. Peyssonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V, Johnson RS: **HIF-1alpha expression regulates the bactericidal capacity of phagocytes**. *J Clin Invest* 2005, **115**(7):1806-1815.
129. Rosenberger CM, Gallo RL, Finlay BB: **Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular Salmonella replication**. *Proc Natl Acad Sci U S A* 2004, **101**(8):2422-2427.
130. Selsted ME, Ouellette AJ: **Mammalian defensins in the antimicrobial immune response**. *Nat Immunol* 2005, **6**(6):551-557.
131. Jones DE, Bevins CL: **Paneth cells of the human small intestine express an antimicrobial peptide gene**. *J Biol Chem* 1992, **267**(32):23216-23225.
132. Jones DE, Bevins CL: **Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel**. *FEBS Lett* 1993, **315**(2):187-192.
133. Svinarich DM, Wolf NA, Gomez R, Gonik B, Romero R: **Detection of human defensin 5 in reproductive tissues**. *Am J Obstet Gynecol* 1997, **176**(2):470-475.
134. Bensch KW, Raida M, Magert HJ, Schulz-Knappe P, Forssmann WG: **hBD-1: a novel beta-defensin from human plasma**. *FEBS Lett* 1995, **368**(2):331-335.
135. Harder J, Bartels J, Christophers E, Schroder JM: **A peptide antibiotic from human skin**. *Nature* 1997, **387**(6636):861.
136. Harder J, Bartels J, Christophers E, Schroder JM: **Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic**. *J Biol Chem* 2001, **276**(8):5707-5713.
137. Garcia JR, Krause A, Schulz S, Rodriguez-Jimenez FJ, Kluver E, Adermann K, Forssmann U, Frimpong-Boateng A, Bals R, Forssmann WG: **Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity**. *FASEB J* 2001, **15**(10):1819-1821.
138. Schutte BC, Mitros JP, Bartlett JA, Walters JD, Jia HP, Welsh MJ, Casavant TL, McCray PB, Jr.: **Discovery of five conserved beta -defensin gene clusters using a computational search strategy**. *Proc Natl Acad Sci U S A* 2002, **99**(4):2129-2133.
139. Duits LA, Ravensbergen B, Rademaker M, Hiemstra PS, Nibbering PH: **Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells**. *Immunology* 2002, **106**(4):517-525.
140. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R: **IL-22 increases the innate immunity of tissues**. *Immunity* 2004, **21**(2):241-254.
141. Harder J, Meyer-Hoffert U, Teran LM, Schwichtenberg L, Bartels J, Maune S, Schroder JM: **Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia**. *Am J Respir Cell Mol Biol* 2000, **22**(6):714-721.
142. Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI: **Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils**. *Antimicrob Agents Chemother* 1998, **42**(9):2206-2214.
143. Bergman P, Johansson L, Asp V, Plant L, Gudmundsson GH, Jonsson AB, Agerberth B: **Neisseria gonorrhoeae downregulates expression of the human antimicrobial peptide LL-37**. *Cell Microbiol* 2005, **7**(7):1009-1017.
144. Bergman P, Walter-Jallow L, Broliden K, Agerberth B, Soderlund J: **The antimicrobial peptide LL-37 inhibits HIV-1 replication**. *Curr HIV Res* 2007, **5**(4):410-415.
145. Yasin B, Pang M, Turner JS, Cho Y, Dinh NN, Waring AJ, Lehrer RI, Wagar EA: **Evaluation of the inactivation of infectious Herpes simplex virus by host-defense peptides**. *Eur J Clin Microbiol Infect Dis* 2000, **19**(3):187-194.
146. Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY: **Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum**. *J Immunol* 2004, **172**(3):1763-1767.
147. Bergman P, Termen S, Johansson L, Nystrom L, Arenas E, Jonsson AB, Hokfelt T, Gudmundsson GH, Agerberth B: **The antimicrobial peptide**

rCRAMP is present in the central nervous system of the rat. *J Neurochem* 2005, **93**(5):1132-1140.
148. Travis SM, Anderson NN, Forsyth WR, Espiritu C, Conway BD, Greenberg EP, McCray PB, Jr., Lehrer RI, Welsh MJ, Tack BF: **Bactericidal activity of mammalian cathelicidin-derived peptides**. *Infect Immun* 2000, **68**(5):2748-2755.
149. Larrick JW, Hirata M, Shimomoura Y, Yoshida M, Zheng H, Zhong J, Wright SC: **Antimicrobial activity of rabbit CAP18-derived peptides**. *Antimicrob Agents Chemother* 1993, **37**(12):2534-2539.
150. Kai-Larsen Y, Luthje P, Chromek M, Peters V, Wang X, Holm A, Kadas L, Hedlund KO, Johansson J, Chapman MR *et al*: **Uropathogenic Escherichia coli modulates immune responses and its curli fimbriae interact with the antimicrobial peptide LL-37**. *PLoS Pathog* 2010, **6**(7):e1001010.
151. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM: **Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis**. *Cell* 1997, **88**(4):553-560.
152. Routsias JG, Karagounis P, Parvulesku G, Legakis NJ, Tsakris A: **In vitro bactericidal activity of human beta-defensin 2 against nosocomial strains**. *Peptides* 2010, **31**(9):1654-1660.
153. Sahl HG, Pag U, Bonness S, Wagner S, Antcheva N, Tossi A: **Mammalian defensins: structures and mechanism of antibiotic activity**. *J Leukoc Biol* 2005, **77**(4):466-475.
154. Kraemer BF, Campbell RA, Schwertz H, Cody MJ, Franks Z, Tolley ND, Kahr WH, Lindemann S, Seizer P, Yost CC *et al*: **Novel anti-bacterial activities of beta-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation**. *PLoS Pathog* 2011, **7**(11):e1002355.
155. Feng Z, Jiang B, Chandra J, Ghannoum M, Nelson S, Weinberg A: **Human beta-defensins: differential activity against candidal species and regulation by Candida albicans**. *J Dent Res* 2005, **84**(5):445-450.
156. Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF, Wehkamp J: **Reduction of disulphide bonds unmasks potent antimicrobial activity of human beta-defensin 1**. *Nature* 2011, **469**(7330):419-423.
157. Quinones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, Marotta ML, Mirza M, Jiang B, Kiser P *et al*: **Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication**. *AIDS* 2003, **17**(16):F39-48.
158. Howell MD, Streib JE, Leung DY: **Antiviral activity of human beta-defensin 3 against vaccinia virus**. *J Allergy Clin Immunol* 2007, **119**(4):1022-1025.
159. Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Keller MJ, Lehrer RI, Herold BC: **Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection**. *J Immunol* 2006, **177**(12):8658-8666.
160. Zaalouk TK, Bajaj-Elliott M, George JT, McDonald V: **Differential regulation of beta-defensin gene expression during Cryptosporidium parvum infection**. *Infect Immun* 2004, **72**(5):2772-2779.
161. Chakraborty K, Ghosh S, Koley H, Mukhopadhyay AK, Ramamurthy T, Saha DR, Mukhopadhyay D, Roychowdhury S, Hamabata T, Takeda Y *et al*: **Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells**. *Cell Microbiol* 2008, **10**(12):2520-2537.
162. Moranta D, Regueiro V, March C, Llobet E, Margareto J, Larrarte E, Garmendia J, Bengoechea JA: **Klebsiella pneumoniae capsule polysaccharide impedes the expression of beta-defensins by airway epithelial cells**. *Infect Immun* 2010, **78**(3):1135-1146.
163. Schmidtchen A, Frick IM, Andersson E, Tapper H, Bjorck L: **Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37**. *Mol Microbiol* 2002, **46**(1):157-168.
164. Belas R, Manos J, Suvanasuthi R: **Proteus mirabilis ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides**. *Infect Immun* 2004, **72**(9):5159-5167.

165. Gutner M, Chaushu S, Balter D, Bachrach G: **Saliva enables the antimicrobial activity of LL-37 in the presence of proteases of Porphyromonas gingivalis**. *Infect Immun* 2009, **77**(12):5558-5563.
166. Sieprawska-Lupa M, Mydel P, Krawczyk K, Wojcik K, Puklo M, Lupa B, Suder P, Silberring J, Reed M, Pohl J *et al*: **Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases**. *Antimicrob Agents Chemother* 2004, **48**(12):4673-4679.
167. Maisetta G, Brancatisano FL, Esin S, Campa M, Batoni G: **Gingipains produced by Porphyromonas gingivalis ATCC49417 degrade human-beta-defensin 3 and affect peptide's antibacterial activity in vitro**. *Peptides* 2011, **32**(5):1073-1077.
168. Thomassin JL, Brannon JR, Gibbs BF, Gruenheid S, Le Moual H: **OmpT outer membrane proteases of enterohemorrhagic and enteropathogenic Escherichia coli contribute differently to the degradation of human LL-37**. *Infect Immun* 2012, **80**(2):483-492.
169. Galvan EM, Lasaro MA, Schifferli DM: **Capsular antigen fraction 1 and Pla modulate the susceptibility of Yersinia pestis to pulmonary antimicrobial peptides such as cathelicidin**. *Infect Immun* 2008, **76**(4):1456-1464.
170. Le Sage V, Zhu L, Lepage C, Portt A, Viau C, Daigle F, Gruenheid S, Le Moual H: **An outer membrane protease of the omptin family prevents activation of the Citrobacter rodentium PhoPQ two-component system by antimicrobial peptides**. *Mol Microbiol* 2009, **74**(1):98-111.
171. Pence MA, Rooijakkers SH, Cogen AL, Cole JN, Hollands A, Gallo RL, Nizet V: **Streptococcal inhibitor of complement promotes innate immune resistance phenotypes of invasive M1T1 group A Streptococcus**. *J Innate Immun* 2010, **2**(6):587-595.
172. Campos MA, Vargas MA, Regueiro V, Llompart CM, Alberti S, Bengoechea JA: **Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides**. *Infect Immun* 2004, **72**(12):7107-7114.
173. Spinosa MR, Progida C, Tala A, Cogli L, Alifano P, Bucci C: **The Neisseria meningitidis capsule is important for intracellular survival in human cells**. *Infect Immun* 2007, **75**(7):3594-3603.
174. Jones A, Georg M, Maudsdotter L, Jonsson AB: **Endotoxin, capsule, and bacterial attachment contribute to Neisseria meningitidis resistance to the human antimicrobial peptide LL-37**. *J Bacteriol* 2009, **191**(12):3861-3868.
175. Kocianova S, Vuong C, Yao Y, Voyich JM, Fischer ER, DeLeo FR, Otto M: **Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of Staphylococcus epidermidis**. *J Clin Invest* 2005, **115**(3):688-694.
176. Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M: **Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system**. *Cell Microbiol* 2004, **6**(3):269-275.
177. Kristian SA, Lauth X, Nizet V, Goetz F, Neumeister B, Peschel A, Landmann R: **Alanylation of teichoic acids protects Staphylococcus aureus against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model**. *J Infect Dis* 2003, **188**(3):414-423.
178. Nishi H, Komatsuzawa H, Fujiwara T, McCallum N, Sugai M: **Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in Staphylococcus aureus**. *Antimicrob Agents Chemother* 2004, **48**(12):4800-4807.
179. Moskowitz SM, Ernst RK, Miller SI: **PmrAB, a two-component regulatory system of Pseudomonas aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A**. *J Bacteriol* 2004, **186**(2):575-579.
180. Tzeng YL, Ambrose KD, Zughaier S, Zhou X, Miller YK, Shafer WM, Stephens DS: **Cationic antimicrobial peptide resistance in Neisseria meningitidis**. *J Bacteriol* 2005, **187**(15):5387-5396.

181. Hamilton A, Popham DL, Carl DJ, Lauth X, Nizet V, Jones AL: **Penicillin-binding protein 1a promotes resistance of group B streptococcus to antimicrobial peptides**. *Infect Immun* 2006, **74**(11):6179-6187.
182. Shafer WM, Qu X, Waring AJ, Lehrer RI: **Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family**. *Proc Natl Acad Sci U S A* 1998, **95**(4):1829-1833.
183. Padilla E, Llobet E, Domenech-Sanchez A, Martinez-Martinez L, Bengoechea JA, Alberti S: **Klebsiella pneumoniae AcrAB efflux pump contributes to antimicrobial resistance and virulence**. *Antimicrob Agents Chemother* 2010, **54**(1):177-183.
184. Shelton CL, Raffel FK, Beatty WL, Johnson SM, Mason KM: **Sap transporter mediated import and subsequent degradation of antimicrobial peptides in haemophilus**. *PLoS Pathog* 2011, **7**(11):e1002360.
185. Goytia M, Shafer WM: **Polyamines can increase resistance of Neisseria gonorrhoeae to mediators of the innate human host defense**. *Infect Immun* 2010, **78**(7):3187-3195.
186. De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O: **LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells**. *J Exp Med* 2000, **192**(7):1069-1074.
187. Kurosaka K, Chen Q, Yarovinsky F, Oppenheim JJ, Yang D: **Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant**. *J Immunol* 2005, **174**(10):6257-6265.
188. Presicce P, Giannelli S, Taddeo A, Villa ML, Della Bella S: **Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91**. *J Leukoc Biol* 2009, **86**(4):941-948.
189. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schroder JM, Wang JM, Howard OM *et al*: **Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6**. *Science* 1999, **286**(5439):525-528.
190. Rohrl J, Yang D, Oppenheim JJ, Hehlgans T: **Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2**. *J Immunol* 2010, **184**(12):6688-6694.
191. Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, Nagaoka I: **A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis**. *Immunology* 2002, **106**(1):20-26.
192. Niyonsaba F, Iwabuchi K, Matsuda H, Ogawa H, Nagaoka I: **Epithelial cell-derived human beta-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway**. *Int Immunol* 2002, **14**(4):421-426.
193. Lai Y, Gallo RL: **AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense**. *Trends Immunol* 2009, **30**(3):131-141.
194. Wan M, Sabirsh A, Wetterholm A, Agerberth B, Haeggstrom JZ: **Leukotriene B4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses**. *FASEB J* 2007, **21**(11):2897-2905.
195. Kandler K, Shaykhiev R, Kleemann P, Klescz F, Lohoff M, Vogelmeier C, Bals R: **The anti-microbial peptide LL-37 inhibits the activation of dendritic cells by TLR ligands**. *Int Immunol* 2006, **18**(12):1729-1736.
196. Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE: **The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses**. *J Immunol* 2002, **169**(7):3883-3891.
197. Kohlgraf KG, Pingel LC, Dietrich DE, Brogden KA: **Defensins as anti-inflammatory compounds and mucosal adjuvants**. *Future Microbiol* 2010, **5**(1):99-113.

198. Nomura I, Goleva E, Howell MD, Hamid QA, Ong PY, Hall CF, Darst MA, Gao B, Boguniewicz M, Travers JB *et al*: **Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes**. *J Immunol* 2003, **171**(6):3262-3269.
199. Christophers E, Henseler T: **Contrasting disease patterns in psoriasis and atopic dermatitis**. *Arch Dermatol Res* 1987, **279 Suppl**:S48-51.
200. Howell MD, Wollenberg A, Gallo RL, Flaig M, Streib JE, Wong C, Pavicic T, Boguniewicz M, Leung DY: **Cathelicidin deficiency predisposes to eczema herpeticum**. *J Allergy Clin Immunol* 2006, **117**(4):836-841.
201. Hata TR, Kotol P, Boguniewicz M, Taylor P, Paik A, Jackson M, Nguyen M, Kabigting F, Miller J, Gerber M *et al*: **History of eczema herpeticum is associated with the inability to induce human beta-defensin (HBD)-2, HBD-3 and cathelicidin in the skin of patients with atopic dermatitis**. *Br J Dermatol* 2010, **163**(3):659-661.
202. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL: **Innate antimicrobial peptide protects the skin from invasive bacterial infection**. *Nature* 2001, **414**(6862):454-457.
203. Braff MH, Zaiou M, Fierer J, Nizet V, Gallo RL: **Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens**. *Infect Immun* 2005, **73**(10):6771-6781.
204. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Su B, Nestle FO, Zal T *et al*: **Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide**. *Nature* 2007, **449**(7162):564-569.
205. Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, Homey B, Barrat FJ, Zal T, Gilliet M: **Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8**. *J Exp Med* 2009, **206**(9):1983-1994.
206. Yamasaki K, Di Nardo A, Bardan A, Murakami M, Ohtake T, Coda A, Dorschner RA, Bonnart C, Descargues P, Hovnanian A *et al*: **Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea**. *Nat Med* 2007, **13**(8):975-980.
207. O'Sullivan BP, Freedman SD: **Cystic fibrosis**. *Lancet* 2009, **373**(9678):1891-1904.
208. Bergsson G, Reeves EP, McNally P, Chotirmall SH, Greene CM, Greally P, Murphy P, O'Neill SJ, McElvaney NG: **LL-37 complexation with glycosaminoglycans in cystic fibrosis lungs inhibits antimicrobial activity, which can be restored by hypertonic saline**. *J Immunol* 2009, **183**(1):543-551.
209. Bals R, Weiner DJ, Meegalla RL, Wilson JM: **Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model**. *J Clin Invest* 1999, **103**(8):1113-1117.
210. Bals R, Weiner DJ, Moscioni AD, Meegalla RL, Wilson JM: **Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide**. *Infect Immun* 1999, **67**(11):6084-6089.
211. Ryan LK, Dai J, Yin Z, Megjugorac N, Uhlhorn V, Yim S, Schwartz KD, Abrahams JM, Diamond G, Fitzgerald-Bocarsly P: **Modulation of human beta-defensin-1 (hBD-1) in plasmacytoid dendritic cells (PDC), monocytes, and epithelial cells by influenza virus, Herpes simplex virus, and Sendai virus and its possible role in innate immunity**. *J Leukoc Biol* 2011, **90**(2):343-356.
212. Putsep K, Carlsson G, Boman HG, Andersson M: **Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study**. *Lancet* 2002, **360**(9340):1144-1149.
213. Gombart AF, Shiohara M, Kwok SH, Agematsu K, Komiyama A, Koeffler HP: **Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein--epsilon**. *Blood* 2001, **97**(9):2561-2567.

214. Brancatisano FL, Maisetta G, Barsotti F, Esin S, Miceli M, Gabriele M, Giuca MR, Campa M, Batoni G: **Reduced human beta defensin 3 in individuals with periodontal disease**. *J Dent Res* 2011, **90**(2):241-245.
215. Fukumoto K, Nagaoka I, Yamataka A, Kobayashi H, Yanai T, Kato Y, Miyano T: **Effect of antibacterial cathelicidin peptide CAP18/LL-37 on sepsis in neonatal rats**. *Pediatr Surg Int* 2005, **21**(1):20-24.
216. Wu M, McClellan SA, Barrett RP, Zhang Y, Hazlett LD: **Beta-defensins 2 and 3 together promote resistance to Pseudomonas aeruginosa keratitis**. *J Immunol* 2009, **183**(12):8054-8060.
217. Schauber J, Svanholm C, Termen S, Iffland K, Menzel T, Scheppach W, Melcher R, Agerberth B, Luhrs H, Gudmundsson GH: **Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways**. *Gut* 2003, **52**(5):735-741.
218. Schauber J, Dorschner RA, Yamasaki K, Brouha B, Gallo RL: **Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli**. *Immunology* 2006, **118**(4):509-519.
219. Schwab M, Reynders V, Shastri Y, Loitsch S, Stein J, Schroder O: **Role of nuclear hormone receptors in butyrate-mediated up-regulation of the antimicrobial peptide cathelicidin in epithelial colorectal cells**. *Mol Immunol* 2007, **44**(8):2107-2114.
220. Schauber J, Iffland K, Frisch S, Kudlich T, Schmausser B, Eck M, Menzel T, Gostner A, Luhrs H, Scheppach W: **Histone-deacetylase inhibitors induce the cathelicidin LL-37 in gastrointestinal cells**. *Mol Immunol* 2004, **41**(9):847-854.
221. Kida Y, Shimizu T, Kuwano K: **Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1**. *Mol Immunol* 2006, **43**(12):1972-1981.
222. Termen S, Tollin M, Rodriguez E, Sveinsdottir SH, Johannesson B, Cederlund A, Sjovall J, Agerberth B, Gudmundsson GH: **PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells**. *Mol Immunol* 2008, **45**(15):3947-3955.
223. Steinmann J, Halldorsson S, Agerberth B, Gudmundsson GH: **Phenylbutyrate induces antimicrobial peptide expression**. *Antimicrob Agents Chemother* 2009, **53**(12):5127-5133.
224. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JW, Mader S *et al*: **Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression**. *J Immunol* 2004, **173**(5):2909-2912.
225. Gombart AF, Borregaard N, Koeffler HP: **Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3**. *FASEB J* 2005, **19**(9):1067-1077.
226. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C *et al*: **Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response**. *Science* 2006, **311**(5768):1770-1773.
227. Schauber J, Oda Y, Buchau AS, Yun QC, Steinmeyer A, Zugel U, Bikle DD, Gallo RL: **Histone acetylation in keratinocytes enables control of the expression of cathelicidin and CD14 by 1,25-dihydroxyvitamin D3**. *J Invest Dermatol* 2008, **128**(4):816-824.
228. Rivas-Santiago CE, Rivas-Santiago B, Leon DA, Castaneda-Delgado J, Hernandez Pando R: **Induction of beta-defensins by l-isoleucine as novel immunotherapy in experimental murine tuberculosis**. *Clin Exp Immunol* 2011, **164**(1):80-89.
229. Talukder P, Satho T, Irie K, Sharmin T, Hamady D, Nakashima Y, Kashige N, Miake F: **Trace metal zinc stimulates secretion of antimicrobial peptide LL-37 from Caco-2 cells through ERK and p38 MAP kinase**. *Int Immunopharmacol* 2011, **11**(1):141-144.

230. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A: **Potential beneficial effects of butyrate in intestinal and extraintestinal diseases**. *World J Gastroenterol* 2011, **17**(12):1519-1528.
231. Mortensen PB, Clausen MR: **Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease**. *Scand J Gastroenterol Suppl* 1996, **216**:132-148.
232. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, Richter F, Dusel G, Kasper H: **Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis**. *Gastroenterology* 1992, **103**(1):51-56.
233. Steinhart AH, Brzezinski A, Baker JP: **Treatment of refractory ulcerative proctosigmoiditis with butyrate enemas**. *Am J Gastroenterol* 1994, **89**(2):179-183.
234. Vernia P, Monteleone G, Grandinetti G, Villotti G, Di Giulio E, Frieri G, Marcheggiano A, Pallone F, Caprilli R, Torsoli A: **Combined oral sodium butyrate and mesalazine treatment compared to oral mesalazine alone in ulcerative colitis: randomized, double-blind, placebo-controlled pilot study**. *Dig Dis Sci* 2000, **45**(5):976-981.
235. Vernia P, Annese V, Bresci G, d'Albasio G, D'Inca R, Giaccari S, Ingrosso M, Mansi C, Riegler G, Valpiani D *et al*: **Topical butyrate improves efficacy of 5-ASA in refractory distal ulcerative colitis: results of a multicentre trial**. *Eur J Clin Invest* 2003, **33**(3):244-248.
236. Vernia P, Fracasso PL, Casale V, Villotti G, Marcheggiano A, Stigliano V, Pinnaro P, Bagnardi V, Caprilli R: **Topical butyrate for acute radiation proctitis: randomised, crossover trial**. *Lancet* 2000, **356**(9237):1232-1235.
237. Luhrs H, Gerke T, Muller JG, Melcher R, Schauber J, Boxberge F, Scheppach W, Menzel T: **Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis**. *Scand J Gastroenterol* 2002, **37**(4):458-466.
238. Di Sabatino A, Morera R, Ciccocioppo R, Cazzola P, Gotti S, Tinozzi FP, Tinozzi S, Corazza GR: **Oral butyrate for mildly to moderately active Crohn's disease**. *Aliment Pharmacol Ther* 2005, **22**(9):789-794.
239. Segain JP, Raingeard de la Bletiere D, Bourreille A, Leray V, Gervois N, Rosales C, Ferrier L, Bonnet C, Blottiere HM, Galmiche JP: **Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease**. *Gut* 2000, **47**(3):397-403.
240. Butzner JD, Parmar R, Bell CJ, Dalal V: **Butyrate enema therapy stimulates mucosal repair in experimental colitis in the rat**. *Gut* 1996, **38**(4):568-573.
241. Klampfer L, Huang J, Sasazuki T, Shirasawa S, Augenlicht L: **Inhibition of interferon gamma signaling by the short chain fatty acid butyrate**. *Mol Cancer Res* 2003, **1**(11):855-862.
242. Yeung AT, Gellatly SL, Hancock RE: **Multifunctional cationic host defence peptides and their clinical applications**. *Cell Mol Life Sci* 2011, **68**(13):2161-2176.
243. Rabbani GH, Albert MJ, Hamidur Rahman AS, Moyenul Isalm M, Nasirul Islam KM, Alam K: **Short-chain fatty acids improve clinical, pathologic, and microbiologic features of experimental shigellosis**. *J Infect Dis* 1999, **179**(2):390-397.
244. Van Vlem B, Vanholder R, De Paepe P, Vogelaers D, Ringoir S: **Immunomodulating effects of antibiotics: literature review**. *Infection* 1996, **24**(4):275-291.
245. Etheridge ME, Hoque AT, Sack DA: **Pathologic study of a rabbit model for shigellosis**. *Lab Anim Sci* 1996, **46**(1):61-66.
246. Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF: **Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens**. *J Immunol* 2005, **174**(8):4901-4907.
247. Gibson PR, Kilias D, Rosella O, Day JM, Abbott M, Finch CF, Young GP: **Effect of topical butyrate on rectal epithelial kinetics and mucosal enzyme activities**. *Clin Sci (Lond)* 1998, **94**(6):671-676.
248. Rabbani GH, Ahmed S, Hossain I, Islam R, Marni F, Akhtar M, Majid N: **Green banana reduces clinical severity of childhood shigellosis: a double-**

blind, randomized, controlled clinical trial. *Pediatr Infect Dis J* 2009, **28**(5):420-425.
249. Egorin MJ, Yuan ZM, Sentz DL, Plaisance K, Eiseman JL: **Plasma pharmacokinetics of butyrate after intravenous administration of sodium butyrate or oral administration of tributyrin or sodium butyrate to mice and rats**. *Cancer Chemother Pharmacol* 1999, **43**(6):445-453.
250. Raqib R, Ljungdahl A, Lindberg AA, Wretlind B, Andersson U, Andersson J: **Dissociation between cytokine mRNA expression and protein production in shigellosis**. *Eur J Immunol* 1996, **26**(5):1130-1138.
251. Hale TL, Formal SB: **Protein synthesis in HeLa or Henle 407 cells infected with Shigella dysenteriae 1, Shigella flexneri 2a, or Salmonella typhimurium W118**. *Infect Immun* 1981, **32**(1):137-144.
252. Mandell LA, Afnan M: **Mechanisms of interaction among subinhibitory concentrations of antibiotics, human polymorphonuclear neutrophils, and gram-negative bacilli**. *Antimicrob Agents Chemother* 1991, **35**(7):1291-1297.
253. Nau R, Eiffert H: **Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis**. *Clin Microbiol Rev* 2002, **15**(1):95-110.
254. Dalhoff A: **Immunomodulatory activities of fluoroquinolones**. *Infection* 2005, **33 Suppl 2**:55-70.
255. Cacchillo DA, Walters JD: **Effect of ciprofloxacin on killing of Actinobacillus actinomycetemcomitans by polymorphonuclear leukocytes**. *Antimicrob Agents Chemother* 2002, **46**(6):1980-1984.
256. Wong JP, Schnell G, Simpson M, Saravolac E: **Effects of liposome-encapsulated ciprofloxacin on phagocytosis, nitric oxide and intracellular killing of Staphylcoccus aureus by murine macrophages**. *Artif Cells Blood Substit Immobil Biotechnol* 2000, **28**(5):415-428.
257. Katsuno G, Takahashi HK, Iwagaki H, Sugita S, Mori S, Saito S, Yoshino T, Nishibori M, Tanaka N: **The effect of ciprofloxacin on CD14 and toll-like receptor-4 expression on human monocytes**. *Shock* 2006, **25**(3):247-253.
258. Kolios G, Manousou P, Bourikas L, Notas G, Tsagarakis N, Mouzas I, Kouroumalis E: **Ciprofloxacin inhibits cytokine-induced nitric oxide production in human colonic epithelium**. *Eur J Clin Invest* 2006, **36**(10):720-729.
259. Dalhoff A, Shalit I: **Immunomodulatory effects of quinolones**. *Lancet Infect Dis* 2003, **3**(6):359-371.
260. Shalit I, Kletter Y, Weiss K, Gruss T, Fabian I: **Enhanced hematopoiesis in sublethally irradiated mice treated with various quinolones**. *Eur J Haematol* 1997, **58**(2):92-98.
261. Yamashita Y, Ashizawa T, Morimoto M, Hosomi J, Nakano H: **Antitumor quinolones with mammalian topoisomerase II mediated DNA cleavage activity**. *Cancer Res* 1992, **52**(10):2818-2822.
262. Perez-Cadahia B, Drobic B, Davie JR: **H3 phosphorylation: dual role in mitosis and interphase**. *Biochem Cell Biol* 2009, **87**(5):695-709.
263. Bode AM, Dong Z: **Inducible covalent posttranslational modification of histone H3**. *Sci STKE* 2005, **2005**(281):re4.
264. Park GY, Joo M, Pedchenko T, Blackwell TS, Christman JW: **Regulation of macrophage cyclooxygenase-2 gene expression by modifications of histone H3**. *Am J Physiol Lung Cell Mol Physiol* 2004, **286**(5):L956-962.
265. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J *et al*: **Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation**. *J Biol Chem* 2003, **278**(28):25481-25489.
266. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D *et al*: **Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43**. *Nature* 2009, **461**(7268):1282-1286.

267. Kanada KN, Nakatsuji T, Gallo RL: **Doxycycline Indirectly Inhibits Proteolytic Activation of Tryptic Kallikrein-Related Peptidases and Activation of Cathelicidin**. *J Invest Dermatol* 2012, **132**(5):1435-1442.
268. Hsu K, Champaiboon C, Guenther BD, Sorenson BS, Khammanivong A, Ross KF, Geczy CL, Herzberg MC: **Anti-Infective Protective Properties of S100 Calgranulins**. *Antiinflamm Antiallergy Agents Med Chem* 2009, **8**(4):290-305.
269. Dyer KD, Rosenberg HF: **The RNase a superfamily: generation of diversity and innate host defense**. *Mol Divers* 2006, **10**(4):585-597.
270. Giesemann T, Guttenberg G, Aktories K: **Human alpha-defensins inhibit Clostridium difficile toxin B**. *Gastroenterology* 2008, **134**(7):2049-2058.
271. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG: **Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits**. *Nature* 2008, **455**(7214):804-807.
272. Gill N, Wlodarska M, Finlay BB: **Roadblocks in the gut: barriers to enteric infection**. *Cell Microbiol* 2011, **13**(5):660-669.
273. Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K, Andersson M: **Secreted enteric antimicrobial activity localises to the mucus surface layer**. *Gut* 2008, **57**(6):764-771.