The Vault

https://prism.ucalgary.ca

Open Theses and Dissertations

2020-03-23

# Immunomodulatory Role of Cathelicidin at Colonic Epithelium

Holani, Ravi

Holani, R. (2020). Immunomodulatory Role of Cathelicidin at Colonic Epithelium (Doctoral thesis, University of Calgary, Calgary, Canada). Retrieved from https://prism.ucalgary.ca. http://hdl.handle.net/1880/111750 Downloaded from PRISM Repository, University of Calgary



# Immunomodulatory Role of Cathelicidin at Colonic Epithelium

by

Ravi Holani

A THESIS

# SUBMITTED TO FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICIAL SCIENCES

CALGARY, ALBERTA

MARCH, 2020

© Ravi Holani 2020

#### ABSTRACT

Cathelicidin, a small, cationic and amphiphilic host defense peptide, is expressed by hematopoietic and non-hematopoietic cells. Initially considered an antimicrobial, cathelicidin are more than simply natural antibiotics. Due to their ability to interact with multiple receptors, cathelicidin can modulate immune responses by a variety of host cells. However, their immunomodulatory effects vary based on their micro-environment, including tissue type (hematopoietic vs. non-hematopoietic), availability of microbes or associated factor(s) and effective concentration of cathelicidin. Since most studies are performed in leukocytes, ability of cathelicidin to regulate immune responses by intestinal epithelium remains largely unknown. The objective was to elucidate immunomodulatory potential of cathelicidin in colonic epithelium, under homeostasis as well as during bacterial colitis. In the first study, we determined how cathelicidin synergized with lipopolysaccharide or Salmonella enterica Typhimurium to enhance production of neutrophil chemokine CXCL8 (human) or murine CXCL1 (functional homologue of CXCL8). Further, we identified a twosignal mechanism triggered by the complex of LPS-LL37, in which nuclear factor kappalight-chain-enhancer of activated B cells (NF-kB) signalling was involved in CXCL8 mRNA synthesis, whereas p38 mitogen-activated protein kinase (p38MAPK) was involved in CXCL8 mRNA stabilization. In a Citrobacter rodentium model of colitis, we corroborated increased synthesis of CXCL1 chemokine, effective neutrophil recruitment and reduced bacterial burden in Camp<sup>+/+</sup> versus Camp<sup>-/-</sup> (cathelicidin null) mice. In the second study, under physiological conditions, cathelicidin induced synthesis of Toll-like receptor (TLR) negative regulator, Toll-interacting protein (Tollip). This induced Tollip production inhibited TLR-mediated apoptosis in colonic epithelium, both in vitro by cytokines tumour necrosis factor-  $\alpha$  and interferon-  $\gamma$  as well as *in vivo* in a *C. rodentium* model of colitis. In conclusion, cathelicidin regulated colonic innate immunity via reduction of unwarranted

i

inflammation by inhibiting TLR responses through Tollip, while promoting a neutrophil response to infection.

# **TABLE OF CONTENTS**

Abstract	i
ist of Tables	iv
ist of Figures	7
Acknowledgements	
Dedication	
Contributions	

Abbreviations

# **Chapter 1. INTRODUCTION**

- Infectious colitis
- LPS as component of Gram negative cell wall
- Colon: anatomy and histology
- Innate immunity at colonic epithelium
- HDPs in the colon
- Cathelicidin
- Cathelicidin gene structure
- Functions of cathelicidin
- Anti-bacterial effects of cathelicidin
- Regulation of apoptosis by cathelicidin
- Immunomodulatory role of cathelicidin
- PRRs
- Cathelicidin in infectious colitis
- Hypotheses & aims

# **Chapter 2. MATERIALS AND METHODS**

- Ethical statement
- Reagents
- Colonic epithelium cell and monocyte culture
- Isolation of murine CECs
- Isolation and culture of colonoids (murine and human)
- Isolation of murine BMMs
- Isolation of human neutrophils
- Bacterial culture
- Host cell stimulation
- Use of pharmacological inhibitors for studying signalling pathways
- Quantification of secreted cytokine
- Protein identification by Western blotting
- mRNA quantification of innate factors
- CXCL8 mRNA stability determination
- Colonic epithelial LPS uptake assay
- Assessing TLR4 location in colonic epithelium
- Plasmid transfection
- Luciferase promoter-based assay
- Human neutrophil calcium flux assay
- NETs quantification
- Mouse experiments
- Ip injection of LPS
- *C. rodentium* infection of mice
- Quantification of secreted proteins
- Protein determination in colonic tissue by Western blotting

- MPO activity assessment
- Histological assessment and immunofluorescence
- Quantification of secreted proteins by multiplex cytokine array
- Bacterial quantification in feces, liver and spleen
- Statistical analyses

# Chapter 3. RESULTS

*STUDY 1.* Cathelicidin triggered neutrophil chemotactic factor CXCL8 synthesis in colonic epithelium in response to Gram negative LPS and promoted early recruitment of neutrophils

- Cathelicidin synergized with *S*. Typhimurium or LPS to stimulate human CXCL8 production.
- LPS and LL-37 through extracellular interaction induced CXCL8 secretion via TLR4.
- LL-37 promoted CXCL8 synthesis via GM1-lipid raft mediated LPS uptake and intracellular TLR4 signalling.
- MEK1/2 and miR-31-mediated NF-κB activation promoted colonic CXCL8 transcriptional production induced by LL-37/LPS.
- LL-37/LPS complex initiated TLR4/Src-EGFR-p38MAPK axis to promote CXCL8 stability.
- Cathelicidin was essential for neutrophil recruitment, activation and pathogen clearance in infectious colitis.

*STUDY 2.* Cathelicidin induced Tollip synthesis and prevented TLR-dependent apoptosis in colonic epithelium

• Cathelicidin prevented apoptosis in colonic epithelium via Tollip.

- Cathelicidin induced Tollip synthesis in hematopoietic and non-hematopoietic cells in colon.
- Cathelicidin prevented IRAK-1 phosphorylation and autocrine expression of proinflammatory cytokines in colonic epithelium via Tollip.
- Cathelicidin promoted Tollip synthesis via EGFR-AGO2 mediated inhibition of micro-RNA-31 maturation.

## **Chapter 4. DISCUSSION**

- Cathelicidin triggered neutrophil chemotactic factor CXCL8 synthesis in colonic epithelium in response to Gram negative LPS and promoted early recruitment of neutrophils
- Cathelicidin induced Tollip synthesis and prevented TLR-dependent apoptosis in colonic epithelium.

# **Chapter 5. CONCLUSIONS AND FUTURE DIRECTION**

## REFERENCES

## **APPENDIX. COPYRIGHT PERMISSIONS**

# LIST OF TABLES

 Table 1.1 Classification of cathelicidin on the basis of structure......

 Table 1.2 Cathelicidin and potential cellular receptor......

 Table 1.3 Cathelicidin inter-talk with various TLRs in the intestinal epithelium......

# LIST OF FIGURES

Figure 1.1 Structure of LPS
Figure 1.2 Interaction of LPS, TLR4 and MD2 and downstream signalling
Figure 1.3 Diagram of colonic mucosa
Figure 1.4 Microscopic anatomy of colon
Figure 1.5 Genetic structure of cathelicidin
Figure 1.6 Cathelicidin and its various receptors
Figure 2.1 Developmental stages of murine and human colonoids
<b>Figure 2.2</b> Gene map for various CXCL8 luciferase promoter constructs and their cloning into pGL3 basic plasmid
Figure 3.1 Chemokine production in CECs upon <i>C. rodentium</i> infection
<b>Figure 3.2</b> Histopathological determination of colitis in <i>Camp</i> <sup>+/+</sup> and <i>Camp</i> <sup>-/-</sup> infected mice
Figure 3.3 Synergistic colonic CXCL8 synthesis by LL-37 and LPS or S. Typhimurium
Figure 3.4 Synergistic colonic CXCL8 synthesis by LL-37 and LPS in colonic epithelium and THP-1 monocytes
<b>Figure 3.5</b> Dose and time curves for CXCL8 synthesis in colonic epithelium and cytokine secretion by BMMs in presence of LPS and/or LL-37
Figure 3.6 TLR4 dependent colonic CXCL8 synthesis by LL-37 and LPS
<b>Figure 3.7</b> Intracellular TLR4 expression and cathelicidin-mediated LPS uptake in HT29 cells
Figure 3.8 Lipid raft dependent LPS uptake in presence of cathelicidin and regulation of CXCL8 synthesis
<b>Figure 3.9</b> NF-κB activation regulated LL-37 and LPS induced colonic <i>CXCL8</i> mRNA synthesis
<b>Figure 3.10</b> MEK1/2 kinase and miR-31-dependent NF-κB activation regulated LL-37 and LPS induced colonic <i>CXCL8</i> mRNA synthesis
<b>Figure 3.11</b> Src-EGFR kinase mediated p38MAPK signalling promoted <i>CXCL8</i> mRNA stabilization in colonic epithelial cells stimulated by cathelicidin and LPS
Figure 3.12 Synergistic CXCL8 synthesis by LPS and cathelicidin in presence of various inhibitors

Figure 3.13 Neutrophil activation and fecal shedding in Camp<sup>+/+</sup> and Camp<sup>-/-</sup> mice infected by C. rodentium..... Figure 3.14 Secreted CXCL8 from colonic epithelium induced calcium flux and activation of human blood-derived neutrophils..... Figure 3.15 Cathelicidin prevented apoptosis in HT29 cells..... Figure 3.16 Cathelicidin prevented apoptosis in  $Camp^{+/+}$  and  $Camp^{-/-}$  mice infected with C. rodentium Figure 3.17 Cathelicidin promoted Tollip protein synthesis..... Figure 3.18 Cathelicidin prevented IRAK-1 phosphorylation and pro-inflammatory cytokine expression in colonic epithelium..... Figure 3.19 Cathelicidin promoted EGFR-AGO2 mediated miR-31 maturation in colonic epithelium..... Figure 3.20 Cathelicidin promoted EGFR-AGO2 mediated miR-31 maturation in colonic epithelium of *Camp*<sup>+/+</sup> mice..... Figure 4.1. Proposed signalling mechanisms elicited by cathelicidin in synergy with LPS to promote CXCL8 secretion in colonic epithelium and subsequent neutrophil recruitment/activation.....

#### ACKNOWLEDGEMENTS

First and foremost, I acknowledge the kindness and support of my supervisor, **Dr Eduardo Cobo**. Beyond his contributions to this thesis he has been more of a friend than a boss. I will cherish all the struggles we have been through together and miss the bittersweet relationship that we shared. I am grateful to Dr. Cobo for making me competent to further my career.

I also thank my present and past committee members, **Dr David Proud**, **Dr. Herman Barkema**, **Dr Hans Vogel**, **Dr Richard Uwiera** and **Dr G. Douglas Inglis**. Without their help, it would have been impossible to complete this thesis on the timeline I followed. I am greatly indebted to them for their valuable comments, suggestions and active participation in the research project. I also extend my heartfelt gratitude to **Dr John Kastelic** for his help in editing this thesis.

A special mention to the most amazing soul on planet earth, **Dr Morley Hollenberg**. He has been a great mentor and his Friday Scottish Club sessions were second-to-none. His dedication and love for science has inspired many students like me. I am grateful to the Hollenberg lab for all their technical support, especially **Dr Mahmoud Saifdeaine** and **Dr Kochiro Mihara**.

I thank the entire Cobo lab for their help over the last four and a half years. I have been really lucky to have co-workers like Paloma Araujo Cavalcante, Yi Lin Tan, Graham Blyth, Anshu Babbar, Cristina Fodor and Priyoshi Lahiri. Each one of them has technically contributed to this thesis. Outside of the lab, I would like to thank all the friends who have become my family in Calgary. It is an exhaustive list but they know who they are.

# DEDICATION

This thesis is dedicated to the best family in the world – my family. They have no clue how much their unfailing support and continuous encouragement for this research work has meant to me. In particular my wife, Anshula Revo, believed in me when, even I, doubted myself. Her relentless love and support cannot be articulated. This thesis is dedicated to the best family in the world which I have.

# CONTRIBUTIONS

# Chapter- 1

- Holani R, Shah C, Haji Q, Inglis GD, Uwiera RR, Cobo ER (2016). Proline-arginine rich (PR-39) cathelicidin: Structure, expression and functional implication in intestinal health. *Comparative Immunology Microbiology Infectious Diseases*, 49: 95-101.
- Holani R, Marin M, Kastelic J, Cobo ER (2018). Antimicrobial peptides in gastrointestinal disease. Chapter "Host defense peptides as innate immunomodulators in the pathogenesis of colitis". Editor: Dr. Chi Hin Cho (Southwest Medical University, Luzhou, China). Elsevier B978-0-12-814319.

# Chapter- 2, 3 and 4

- Holani R, Babbar A, Blyth GAD, Lopes F, Jijon H, Mckay DM, Hollenberg MD, Cobo ER (2020). Cathelicidin-mediated lipopolysaccharide signalling via intracellular TLR4 in colonic epithelial cells evokes CXCL8 production. *Gut Microbes*. (under revision)
- Holani R, Babbar A, Blyth GAD, Hollenberg MD, Cobo ER (2020). Cathelicidin promotes Tollip synthesis and prevents apoptosis in colonic epithelium. (In preparation)
- \*Marin M, \*Holani R, Shah C, Odeon A, Cobo ER (2017). Cathelicidin modulates synthesis of Toll-like Receptors (TLRs) 4 and 9 in colonic epithelium. *Molecular Immunology*, 91: 249-258. \*Both authors contributed equally.

## Miscellaneous (not included in thesis)

- \*Cobo ER, \*Holani R, Moreau F, Nakamura K, Ayabe T, Mastroianni J, Ouellette A, Chadee K (2018). *Entamoeba histolytica* alters ileal Paneth cell functions in intact and Muc2 mucin deficiency. *Infection and Immunity*, 86, 7: 1-16. Selected for "*Spotlight*" as research article of significant interest from the current issue. \*Both authors contributed equally.
- Marin M, Holani R, Blyth GAD, Drouin D, Odeón A, Cobo ER (2019). Human cathelicidin improves colonic epithelial defenses against *Salmonella typhimurium* by modulating bacterial invasion, TLR4, and pro-inflammatory cytokines. *Cell and Tissue Research*, 376(3):433-442.
- Cobo ER, Kissoon-Singh V, Moreau F, **Holani R**, Chadee K (2017). MUC2 mucin and butyrate contribute to the synthesis of the antimicrobial peptide cathelicidin in response to *Entamoeba histolytica* and dextran sodium sulfate-induced colitis. *Infection and Immunity*, 85, 3 e-00905-16.
- Boucher E, Marin M, Holani R, Moore DP, Cobo ER (2017). *Neospora caninum* infects peripheral blood monocytes and induces characteristic pro-inflammatory cytokines and host defense cathelicidin peptide. *Parasitology Journal*, 24: 1-14.

#### **ABBREVIATIONS**

AGO2- Argonaute 2

ANOVA- One-way analysis of variance

AP-1- Activator protein-1

BMM- Bone marrow-derived macrophage

C. rodentium - Citrobacter rodentium

CAMP- Cationic antimicrobial peptide

CATH-2- Chicken cathelicidin-2

CD14- Cluster of differentiation 14

**CECs-** Primary colonic epithelial cells

CFU- Colony forming units

CRAMP- Cathelicidin-related-antimicrobial-peptide

CXCR2- Cysteine-X-cysteine receptor 2

**DAPI-** 4', 6-diamidino-2- phenylindole

DRs- Death receptors

**DSS**- Dextran-sodium sulphate

**DTT-** Dithiothreitol

EDTA- Ethylenediaminetetraacetic acid

EGFR- Epidermal growth factor receptor

**ERK-** Extracellular signal-regulated kinase

Fas- First apoptotic signal

FasL- First apoptotic signal Ligand

FPRL1/FPR2- Formyl peptide receptor-like-1/Formyl peptide receptor 2

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GM1- Monosialotetrahexosylganglioside

GPCR- G-protein coupled receptor

H&E- Hematoxylin & Eosin

HDP- Host defense peptides

HRP- Horseradish-peroxidase-conjugate

HT29/T84/Caco2- Human colorectal adenocarcinoma cell line/ Colonic epithelial cells

IBD- Inflammatory bowel diseases

IFN- $\gamma$ - Interferon-  $\gamma$ 

IL-1R- Interleukin-1 receptor

IRAK-1- Interleukin-1 receptor-associated kinase 1

IRAK-M-IL-1 receptor associated kinase-M

LBP- Lipid-binding protein

LDH- Lactate dehydrogenase

LL-37- Leucine-leucine 37 amino acid human cathelicidin peptide

LPS- Lipopolysaccharide

LPS-RS- LPS isolated from Rhodobacter sphaeroides

Ly6G- Lymphocyte antigen 6 complex locus G6D

- MAPK- Mitogen-activated protein kinase
- MCP-1- Monocyte chemoattractant protein-1
- MD-2- Myeloid differentiation factor-2
- mDCs- Myeloid derived dendritic cell
- MEK1/2- Mitogen-activated protein kinase kinase
- MFI- Mean fluorescent intensity
- miR-31- Processed form of precursor micro RNA-31 (mir-31)
- MoI- Multiplicity of infection
- MPO- Myeloperoxidase
- MrgX2- Mas-related gene X 2
- MyD88- Myeloid differentiation 88
- NETs- Neutrophil-extracellular-traps
- NFIL-6- Nuclear factor for IL-6 expression
- NF- $\kappa B$  Nuclear factor kappa-light-chain-enhancer of activated B cells
- **ODN** Oligodinucleotide
- **P2X7** Purinoreceptor 7
- PAMP/MAMP- Pathogen/Microbe-associated molecular patterns
- PARP- Poly (ADP-ribose) polymerase
- PBMC- Peripheral blood-derived mononuclear cells

**PBS-Tw-** PBS with 0.05% Tween-20

pDCs- Plasmacytoid dendritic cells

PFA- Paraformaldehyde

PMA- Phorbol 12-myristate 13-acetate

PMAP-36- Porcine myeloid antimicrobial peptide-36

**PPARγ-** Peroxisome proliferator-activated receptor-γ

PR-39- Proline-arginine 39 amino acid cathelicidin peptide

PRR- Pattern recognition receptor

qPCR- Quantitative real time polymerase chain reaction

**RLU-** Relative light units

S. Typhimurium- Salmonella enterica Typhimurium

SEMs- Standard errors of the mean

SIGIRR- Single immunoglobulin interleukin-1 receptor-related protein

sLL-37- scrambled LL-37

Src- Sarcoma kinase

TIR- Toll/IL-1R homology

TLR- Toll-like receptor

TNFR- TNF receptor

**TNF-** $\alpha$ - Tumour necrosis factor- $\alpha$ 

Tollip- Toll-interacting protein

**TRAF6-** Tumour necrosis factor receptor-associated factor 6

**TRIF**- TIR-domain-containing adaptor-inducing interferon  $\beta$ 

VEGF- Vascular endothelial growth factor

WGA- Wheat germ agglutinin

#### **CHAPTER 1- INTRODUCTION**

#### **1.1. Infectious colitis**

Infectious colitis is acute inflammation of the colon caused by any of a number of incitants, including bacteria (e.g., *Salmonella enterica* Typhimurium), protozoa (e.g., *Entamoeba histolytica*, *Crytosporidium parvum*) and viruses (e.g., *cytomegalovirus*).

These pathogens are food-borne and thus, are transmitted to humans via food products contaminated with feces from infected animals or humans. According to the World Health Organisation (WHO), ~550 million cases of food-borne diarrheal diseases are reported each year, with ~230,000 deaths worldwide, of which approximately 30% cases involve children <5 y. The WHO has estimated that food borne infections are highly contagious, with a likelihood of infection of 1 in every 10 individuals. The global burden due to food-borne diarrheal diseases in 2010 was approximately 18 million disability-adjusted life years (DALY; years lost due to mortality + years lost of healthy life due to morbidity), 50% of which were due to non-typhoidal *S. enterica*. Despite continued preventive and curative efforts, infectious colitis still represents a major socio-economical threat to society, especially in the developing world.

For the purpose of this thesis, I will focus only on infectious colitis caused by Gram negative enteric bacteria. Enteric bacterial pathogens initially colonize the intestinal epithelium and then infiltrate into the mucosa, disrupting tight junctions and causing loss of epithelial integrity. Clinically, these infections are associated with excessive fluid loss (diarrhea), abdominal pain, tenesmus, and occasionally rectal bleeding<sup>1</sup>. Although diarrheic colitis is self-limiting in most cases, infected immunocompromised patients, children, or patients with pre-existing medical conditions (e.g., leukemia) can develop severe dehydration and

septicaemia<sup>2</sup>. Infectious colitis caused by *S*. Typhimurium and *Escherichia coli* also infects production animals, including growing calves and piglets<sup>3</sup>. This represents a major foodborne risk, as bacteria in the gastrointestinal tract of livestock can contaminate carcasses during slaughter and meat processing and be a source of contamination for people.

The current treatment for infectious colitis is broadly divided into two types: first, palliative care/symptomatic treatment, namely oral rehydration therapy with glucose electrolyte solutions and use of anti-motility/diarrheal drugs e.g. atropine; and second, antibiotic(s) therapy. Besides being expensive, antibiotic therapy is not effective against all colitis-causing bacterial pathogens<sup>4</sup>. Improper use of antibiotics increases risks of persistent infection(s) as well as, of development of multi-drug resistant bacteria/superbugs, e.g. strains of *S*. Typhimurium and *E. coli*<sup>5, 6</sup>. Furthermore, use of antibiotics may result in non-specific killing of commensals (dysbiosis) and reduced expression of naturally produced antimicrobial factors in intestine<sup>7, 8</sup>. As such, there is an urgent need for novel, cost-effective and efficacious therapies that can target colonic pathogens in a multi-functional way. In this thesis work, we aimed to elucidate immunomodulatory mechanisms of a naturally produced anti-microbial host defense peptides (HDPs), cathelicidin, at the colonic epithelium. This thesis advances our understanding of how cathelicidin regulates innate immunity at the colonic mucosa and suggests that this peptide has potential as a therapeutic against bacterial colitis, as it has both antimicrobial and immunomodulatory properties.

#### 1.1.2. Lipopolysaccharide (LPS) as a component of Gram negative cell walls

Virulence factors are molecules produced by bacteria in order to achieve three main objectives: host invasion, disease manifestation due to exaggerated immune response and evasion of host defenses<sup>9</sup>. Toxins are well known virulence factors of Gram-negative

pathogens. These toxins are broadly classified into two types: endotoxins (LPS) and exotoxins (thermolabile enterotoxin and cholera toxin from non-typhoidal *Salmonella spp*. and *Vibrio cholera*, respectively)<sup>10</sup>. For purpose of the work described in this thesis, I will focus on LPS endotoxin.

Endotoxin LPS is a glycolipid macromolecule and a common surface-associated virulence factor in Gram negative bacteria. LPS comprises ~75% of the outer membrane of such bacteria and has three components: a hydrophobic domain known (lipid A), a non-repeating outer and inner core oligosaccharide and a distal *O*-antigen polysaccharide (**Fig 1.1**).



**Figure 1.1.** Diagrammatic representation of LPS structure as outer O-antigen, a non-repeating core oligosaccharide and a membrane bound lipid A endotoxin. Image courtesy: Online textbook of bacteriology by Dr Kenneth Todar (http://textbookofbacteriology.net/endotoxin.html).

Lipid A is a glucosamine disaccharide ( $\beta$ -glucosamine-(1 $\rightarrow$ 6)- glucosamine-1-phosphate)based lipid that serves as an anchor of LPS, mainly through electrostatic and hydrophobic interactions. The hexa-acylated structure of lipid A ensures decreased membrane fluidity as compared to other cellular membranes. Lipid A is a moderately diverse molecule with a variable number of fatty-acid side chains and terminal phosphate residues. Lipid A is key for innate immune detection of LPS; therefore, modifications of lipid A often occur in pathogenic bacteria as a strategy to evade host immune response. For instance, *S*. Typhimurium activates a two-component environmental sensor-kinase regulator, called PhoP-PhoQ, in the presence of low concentrations of  $Mg^{2+}$ , a condition encountered inside phagolysosomes. PhoP-PhoQ allows modification of the lipid A moiety by addition of palmitate (a fatty acid), favoring evasion from host immune effectors. Lipid A is covalently attached to the inner core oligosaccharide which is in close proximity of the hydrophobic membrane. The inner core mainly consists of 1-4 sugar molecules of 3-deoxy- $\alpha$ -D-mannooctulosonic acid. Another sugar in the inner core is heptulose monosaccharide which is highly phosphorylated (anionic charge) and thus provides membrane stability to bacteria by interacting with Ca<sup>2+</sup> and Mg<sup>2+</sup> cations.

The *O*-antigen is the outermost part of the LPS that faces the extracellular environment and acts as a hydrophilic coating surface. The *O*-antigen is mainly composed of multiple units of oligosaccharides (e.g. glucose, galactose and N-acetylglucosamine), wherein each unit contains 2 to 6 sugar residues. *O*-antigen is the largest part of the LPS molecule and is extremely diverse among strains of same species. Thus, *O*-antigen is commonly used to characterize bacterial colonies. Based on its appearance, a mutant bacterial colony that does not have an *O*-antigen is termed "rough", whereas one that expresses LPS with *O*-antigen is referred to as "smooth". Both inner core and outer *O*-antigen domains tightly pack together by intercalating divalent positively charged ions which help in preventing diffusion of small hydrophobic molecules. Moreover, a close association between polar oligosaccharides (inner and outer layers) and hydrophobic lipid A moiety in LPS molecule helps maintain bacterial integrity<sup>11-13</sup>.

LPS can initiate an immune response in the host through interactions with a series of receptors, including LPS binding protein (LBP), cluster of differentiation (CD) 14, myeloid differentiation (MD)-2 and Toll-like receptor (TLR) 4. In this interaction, LPS from the outer

membrane of the intact Gram negative bacteria or released from vesicles of bacterial origin is initially bound to LBP. Next, LPS micelles are broken down into monomers by CD14 which exists either in solution or bound to the cell membrane via a glycosylphosphatidylinositol anchor. Subsequently, LPS monomers are presented to a heterodimer of TLR4-MD2 and these dimers aggregate and facilitate downstream signalling (**Fig. 1.2**). The structure of TLR4 can be divided into three parts: a leucine-repeat-rich extracellular domain, a transmembrane domain and a Toll/IL-1R homology (TIR) domain-containing cytoplasmic region. The extracellular TLR4 domain provides the necessary charge-based hydrophilic interaction with co-factor MD-2, the main LPS binding module in the TLR4-MD-2 complex. This extracellular domain also facilitates receptor (TLR4-MD-2) oligomerization upon LPS binding (with a TLR4: MD-2: LPS ratio of 2:2:2) (**Fig 1.2**).



**Figure 1.2.** Recognition of LPS micelles by LBP, followed by CD14 mediated isolation of LPS monomers, formation of TLR4-MD-2-LPS complex (2:2:2) and initiation of downstream signalling. Figure adapted from <sup>14</sup>. TLR downstream signalling may involve two pathways: Myeloid differentiation (MyD) 88 dependent and MyD88 independent/ TIR-domain-containing adaptor-inducing interferon  $\beta$  (TRIF) dependent. Signalling through MyD88 promotes nuclear localization of nuclear factor kappa-light-chain-enhancer of

activated B cells (NF-κB) and/or activator protein (AP)-1 transcription factors, whereas TRIF signalling induces nuclear import of IRF-3 transcription factor. Thereafter, these transcription factors drive expression of pro-inflammatory genes.

The single transmembrane domain aids in anchoring the TLR4 protein as well as in receptor oligomerization. The cytoplasmic TIR domain recruits adaptor proteins, including MyD88, TIR domain-containing adaptor protein; also known as Mal, MyD88-adapter-like, TRIF, TRIF-related adaptor molecule, and sterile- $\alpha$ -and-HEAT-Armadillo motifs-containing protein. These adaptor proteins activate various transcription factors, primarily NF- $\kappa$ B and promote a number of physiological outcomes, including cytokine production, proliferation, cell migration and differentiation<sup>15-17</sup> (**Fig 1.2**).

#### 1.2. The colon

#### 1.2.1 Colon anatomy and histology

The colon constitutes a substantial part of the large intestine and is divided into four sections: ascending colon (joins the cecum and goes up the abdomen), transverse colon (across the width of the abdominal cavity), descending colon (along posterior abdominal wall) and sigmoid colon (S-shaped; continuing to end of colon where it connects with rectum). The human colon is ~5 feet long and mostly involved in water and nutrient absorption. It leads into the rectum, a 6-inch long tube that stores feces and terminates in the anus (**Fig 1.3**).



Figure 1.3 Sections of large intestine. Image adapted from A.D.A.M Images.

Histologically, the colon has four fundamental layers: epithelium formed by flat surfaces with invagination (crypts), lamina propria, muscularis mucosa and outer serosa or peritoneum (Fig **1.4**)<sup>1</sup>.



Figure 1.4. Microscopic anatomy of colon. Adapted from General Histologic Anatomy of The Tubular Digestive Tract by Ryan Jennings and Chris Premanandan<sup>2</sup>.

Crypts are lined by four major cell types: stem cells, enterocytes, mucus secreting goblet cells and hormone secreting enteroendocrine cells. Under normal physiological conditions, stem

<sup>&</sup>lt;sup>1</sup> Book- 'Regulation of Gastrointestinal Mucosal Growth' by Jaladanki N. Rao and Jian-Ying Wang <sup>2</sup> Chapter 8- Gastrointestinal System, from book titled- 'General Histologic Anatomy of The Tubular Digestive Tract by Ryan Jennings and Chris Premanandan'

cells are at the bottom of the crypt and undergo fission to generate mitotic stem cells and terminally differentiated cells. Mitotic stem cells reside at the bottom of the crypt, whereas terminally differentiated cells migrate towards the luminal surface and are finally extruded from the mucosal surface between crypts<sup>18, 19</sup>. The flat surface is mainly composed of thin brush-bordered differentiated columnar enterocytes. Complete renewal of colonic crypt to surface epithelial cells usually takes 3 to 8 days<sup>20</sup>. Functionally, the colonic epithelium coexists constantly with a dense and complex milieu of commensal microorganisms or their associated factors called, pathogen-associated molecular patterns (PAMPs). The lamina propria is a layer of connective tissue comprised of mainly type I and type III collagen, as well as elastic fibres, and contains fibroblasts, tissue resident macrophages, plasma cells, mast cells and lymphocytes. The lamina propia also supports an extensive network of lymphatic vessels, nerves and nerve endings, running between epithelial ridges. The lamina propria attaches to the muscularis mucosa, which consists of relatively thin inner circular and outer longitudinal layers (also called taeniae coli) of smooth muscle. The two layers of muscularis mucosa are tightly coiled; their function is to provide flexibility and support to the mucosa and to propel food through the gastrointestinal tract by generating coordinated peristaltic movements through the myenteric plexus (located between circular and longitudinal muscles). The longitudinal layer of muscles are separated from the serosa layer by a layer of loose connective tissue. The serosa (also called the mesothelium) represents the outermost protective layer of squamous epithelial cells; its most important function is to act as a barrier to external environment and limit intestinal damage from spreading to other organs<sup>19, 21</sup>.

#### 1.2.2 Innate immunity at colonic epithelium

The colon is protected by the innate immune system, which includes an anatomical (mucus) and physiological barrier (epithelium). In addition, there is an ancient defense system across living species composed of cellular (i.e., neutrophils and macrophages) and soluble (i.e., antimicrobial peptides and complement protein) components. Initially regarded as immunologically inert, the colonic epithelium has now been unanimously accepted as an integral component of gut innate immunity<sup>22</sup>. The sentinel role of the colonic epithelium is well represented by the wide variety of immune effectors produced, including cationic HDPs (e.g., cathelicidin and defensins) and expression of immune receptors, namely pattern recognition receptor (PRR), including TLRs for sensing microbes or PAMPs.

#### **1.3. HDPs in the colon**

HDPs are a family of evolutionarily conserved, small cationic amphiphilic peptides. They are widespread across the animal kingdom and are also present in plants. In humans, there are two major classes of HDPs: cathelicidin and defensins. For the purpose of this thesis, the focus will be on cathelicidin.

#### 1.3.1 Cathelicidin

Cathelicidin are a major subgroup of mammalian HDPs with both antimicrobial and immunoregulatory activities. These are produced by a variety of cell types, including macrophages, keratinocytes, endothelial cells, epithelial cells and neutrophils<sup>23, 24</sup>. The number of cathelicidin per animal species is variable. Although humans have only one cathelicidin (i.e., the 37-amino acid cathelin-associated peptide with amino terminal sequence Leu-Leu; LL-37) encoded by the *cationic antimicrobial peptide* (*CAMP*) gene, other animals, e.g. cattle and pigs, express several cathelicidin peptides<sup>25</sup>. Cathelicidin peptides are highly

diverse in terms of their sequence, structure and size<sup>23</sup> (**Table 1.1**). On the basis of structure cathelicidin can be divided into three major types-  $\alpha$ -helical,  $\beta$ -hairpin and extended peptides (**Table 1.1**).

Structure	Name	Species	Sequence
α-helical	LL-37	Human	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES
	Cathelicidin- related- antimicrobial peptide (CRAMP)	Murine	GLLRKGGEKIGEKLKKIGQKIKNFFQKLV PQPE
	RL-37	Rhesus monkey	RLGNFFRKVKEKIGGGLKKVGQKIKDFLG NLVPRTAS
	Porcine Myeloid Antimicrobial Peptide-36	Porcine	GRFRRLRKKTRKRLKKIGKVLKWIPPIVG SIPLGC
β-hairpin	Protegrin-1	Porcine	RGGRLCYCRRRFCVCVGR
	Protegrin-2	Porcine	RGGRLCYCRRRFCICV
	Protegrin-3	Porcine	RGGGLCYCRRRFCVCVGR

**Table 1.1** Classification of cathelicidin on the basis of structure.

	Bactenecin- 5	Bovine	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPP FRPPLGPFP
Extended	Proline- arginine 39 amino acid cathelicidin peptide (PR- 39)	Porcine	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPP RFPPRFP
	Tritrpticin	Porcine	VRRFPWWWPFLRR
	Indolicidin	Bovine	ILPWKWPWWPWRR

Closely related peptides such as mouse cathelicidin CRAMP and human cathelicidin LL-37 are both structurally (net positive charge i.e. +6 and  $\alpha$ -helical structure) as well as functionally similar i.e. both peptides have comparable antimicrobial and immunomodulatory functions<sup>25</sup>. On the other hand, cathelicidin peptides with varied structures and sequences might also have broad functional similarities for example, tryptophan-proline riche bovine indolicidin and arginine rich porcine protegrin-1 are both anti-fungal in nature <sup>23</sup>. Thus, despite high intra- and inter-species diversity in cathelicidin peptides, they are broadly conserved in their functional abilities.

#### **1.3.2** Cathelicidin gene structure

The cathelicidin gene is composed of four exons and three introns. The high degree of conservation of intron sequences among distinct cathelicidin play a role in up-regulating their

own transcription<sup>26</sup>. For instance, the second intron of LL-37 induced LL-37 expression levels in luciferase reporter assays<sup>27</sup>. Cathelicidin are synthesized as a precursor called a prepropeptide; it is only after multiple cleavage steps that mature protein is formed. The prepropeptide has an N-terminal domain containing a signal peptide, a well-conserved central cathelin domain and a variable C-terminal domain<sup>28</sup>. Although not contained in the mature peptide, the cathelin domain comprises the largest part of the prepropeptide. In fact, the name 'cathelicidin' is derived from its high homology to cathelin, a cathepsin L inhibitor. The cathelin domain refers to the highly conserved residue sequence coded primarily by the first three exons, a domain shared among all mammalian cathelicidin. The first exon codes for a short untranslated region, a 29-30 amino acid residue signal sequence and the first 37 residues of the cathelin domain. The second and third exons encode the next 60 amino acids of the cathelin pro-peptide. The last exon is translated to yield the final four residues of the cathelin domain and the mature cathelicidin peptide (12-100 amino acid residues) that is cleaved from the prepropeptide by proteases (e.g., elastases). Mature, biologically active cathelicidin peptides can acquire  $\alpha$ -helical (human LL-37 and murine CRAMP),  $\beta$ -hairpin (porcine protegrin) or unconventional confirmations, e.g. type II poly-L-proline helix conformation (porcine PR-39)<sup>23, 25</sup> (Fig 1.5).



**Figure 1.5.** General structure of cathelicidin gene and precursor peptides that include multiple exons translated to a prepropeptide with an N-terminal endoplasmic reticulum targeting sequence. Adapted from Huttner and Bevins, 1999<sup>29</sup>.

#### 1.3.3 Functions of cathelicidin

Cathelicidin are pleiotropic peptides with multiple functions in microbes and mammals. In

this thesis, anti-bacterial action, anti-apoptotic and immunomodulatory roles are emphasized.

#### 1.3.3.1 Anti-bacterial effects of cathelicidin

Cathelicidin have broad spectrum anti-bacterial activity against both Gram negative (e.g., *Enterococcus faecalis*<sup>30</sup>, *Salmonella spp*.<sup>31</sup>, *E. coli*<sup>32</sup> and *Bacillus subtilis*<sup>33</sup>) and Gram positive (e.g., methicillin-resistant *Staphylococcus aureus*<sup>34</sup> and *Streptococcus spp*.<sup>35</sup>) bacteria. The optimal anti-bacterial concentrations of cathelicidin, as they vary across studies, depending on the bacterium used, colony forming units (CFU) of the bacteria, the medium used for the killing assay and the origin of the cathelicidin peptide. As as example, the minimum bactericidal concentrations of variable cathelicidin (porcine myeloid antimicrobial

peptide-36, human LL-37 and chicken CATH-2) required for killing *E. coli* O78 (1 x  $10^{6}$  CFU) ranged from 5 to 10  $\mu$ M (25 to 50  $\mu$ g/mL)<sup>32</sup>. Cathelicidin interact with negatively/anionic charged bacterial membranes to induce membrane perturbations and lysis of the bacterial cell by hydrophobic residues. Another microbicidal role is associated with the porcine PR-39 cathelicidin; a peptide that can penetrate the outer membrane of *E. coli* K12, leading to inhibition of bacterial processes, e.g. DNA replication and protein synthesis<sup>36</sup>. A synthetic analogue derived from porcine cathelicidin PR-39 (i.e., PR-26) kills *S. typhi* and thus, prevents cellular invasion in murine intestinal epithelial (IEC-6) cells<sup>37</sup>. Likewise, LL-37 or its truncated variant LL-31 can kill Gram negative *Burkholderia pseudomallei*, a bacterium that causes meliodosis in Southeast Asia and Northern Australia<sup>38</sup>.

Bactericidal effects of cathelicidin have been demonstrated in animal models. Exogenous PR-39 cathelicidin or its overexpression under the influence of the K-14 promoter in transgenic mice reduced the number of group A *Streptococcus* on the skin of mice<sup>35</sup>. Prophylactic treatment of ova with a D analogue of chicken CATH-2 reduced morbidity from avian *E. coli* infections in chickens challenged 7 days after hatching<sup>39</sup>. It is unclear whether these beneficial roles were due to bacterial killing or to enhancement of immune inflammatory defenses. In that regard, relevance of antimicrobial activity of cathelicidin *in vivo* is not fully understood, as bactericidal activities *in vitro* of these peptides are dependent on surrounding ions and killing buffer (low ionic strength) components. For example, microbicidal activity of cathelicidin against *E. coli* D21 is reduced in high salt concentrations (100 mM), as it distorts the  $\alpha$ -helical structure<sup>40</sup>. Similarly, killing activity of cathelicidin against group B *Streptococcus spp.* (encapsulated and non-encapsulated) and *Pseudomonas aeruginosa* was reduced (by 50 and 90%, respectively) in the presence of lung surfactant<sup>41</sup> and artificial tears<sup>42</sup>. Presence of serum proteins also reduces cathelicidin's antimicrobial activity<sup>40, 43, 44</sup>. Mechanistically, serum protein(s) interacts with the N-terminus of LL-37 peptide, as Nterminal truncated LL-37 variants, e.g. fragment 106 (devoid of first two amino acids) and fragment 110 (devoid of first 6 amino acids), efficiently kills both Gram positive (*Staphylococcus aureus*) and negative bacteria (*E. coli*)<sup>43</sup>. Overall, cathelicidin have antibacterial properties and their broad bacterial targets make them substantially more potent than conventional antibiotics (most have a single mechanism of action). However, this killing activity could be restricted to singular environments (e.g., bottom of intestinal crypts, proximity to Paneth cells) or other similar locations where relatively high cathelicidin concentrations are achieved.

#### 1.3.3.2. Regulation of apoptosis by cathelicidin

Apoptosis is programmed cell-death that is responsible for cell turnover without accompanying inflammation. Apoptosis is broadly classified into two pathways: the extrinsic (DR) pathway and the intrinsic (mitochondrial) pathway. The extrinsic apoptotic pathway is initiated by activation of death receptors (DRs), members of tumour necrosis factor receptor (TNFR) family, e.g.TNFR1 and first apoptotic signal (Fas). In presence of an extrinsic signal(s), such as bacterial infection or LPS, cytotoxic cells upregulate expression of DR ligands such as  $TNF\alpha$  and Fas ligand (FasL)<sup>45</sup>.  $TNF\alpha$  and/or FasL can bind their respective DRs i.e. TNFR1 and Fas to form death-inducing signalling complexes by recruiting caspase-8 and an adaptor protein called Fas associated death domain, eventually leading to cell death<sup>46</sup>. In contrast, the intrinsic pathway of apoptosis is mostly invoked by cell intrinsic stress signals, e.g. DNA damage, hypoxia or toxins<sup>47</sup>. Regardless of cell type, initiation of intrinsic apoptosis results in mitochondrial outer membrane permeabilization, with cytochrome C leaking into the cytoplasm and recruiting apoptosis protease activating factor -1 and caspase-9 to form an 'apoptosome,' followed by execution of apoptosis<sup>47</sup>. Regardless of the initiating

pathway, activation of apoptosis inducts a cascade of events, wherein executioner caspases, e.g. caspase-3, -6 and -7, degrade the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) and promote DNA fragmentation, leading to cell death.

In intestinal epithelial defences, dysregulated apoptosis in colonic epithelium is a pathological hallmark of infectious colitis induced by enteric bacteria such as Shigella *flexneri*<sup>48</sup>, *E. coli*<sup>49</sup> and *Citrobacter rodentium*<sup>50</sup>. Equally important, inflammatory bowel diseases (IBD), e.g. ulcerative colitis, is characterized by uncontrolled apoptotic death of enterocytes<sup>51, 52</sup>, particularly in active crypts<sup>53</sup>. Increased apoptosis leads to a functional loss of the colonic epithelium, resulting in characteristic symptoms of colitis (i.e. poor water absorption/diarrhea and an impaired gut epithelial barrier)<sup>54</sup>. Host cell apoptosis during a bacterial infection can originate from either pathogen-encoded pro-apoptotic factors or hostderived pro-inflammatory cytokines. In this regard, enteric bacteria such as S. flexneri<sup>55</sup>, S. Typhimurium<sup>56</sup> and *Yersinia enterocolitica*<sup>57</sup>, via their type III secretion system, inject virulence factors Ipa B, Sip B and YopP, respectively, into macrophages to induce caspasedependent apoptosis<sup>55-57</sup>. Additionally, Salmonella dublin, Y. enterocolitica and Shigella dysenteriae induce production of pro-apoptotic cytokine TNF-a in HT29, T84 and Caco-2 cells<sup>58</sup>. Likewise, C. rodentium infection enhances pro-inflammatory TNF-α and interferon (IFN)- $\gamma$  in distal colons of infected C57BL/6 mice<sup>59</sup>. These cytokines enhance apoptotic cell death in intestinal (INT407) cells<sup>60</sup>. In this regard, anti-TNF- $\alpha$  therapy reduces ileal<sup>61</sup> and colonic epithelial apoptosis<sup>62</sup> in murine model of experimental colitis (i.e., Crohn's diseaselike ileitis model (SAMP1/YitFc mice)<sup>61</sup> and dinitrobenzenesulfonic acid -induced colitis)<sup>62</sup>, respectively.

Cathelicidin actively participate in regulation of apoptotic cell death, in a concentrationdependent manner. Although cathelicidin concentrations at the intestinal mucosal surface are unknown, physiological concentrations of cathelicidin in bronchial alveolar lavage of infants are under 20  $\mu$ g/mL (4  $\mu$ M)<sup>63</sup>. At these low physiological concentrations (4  $\mu$ M), cathelicidin are anti-apoptotic, as intra-peritoneal injection of LL-37 prevented LPS binding to TLR4 and thus, prevented apoptosis in endothelium of C57BL/6 mice<sup>64</sup>. Likewise, LL-37 enhanced anti-apoptotic protein Bcl-x(L) expression and reduced spontaneous caspase-3 cleavage via signalling by FPR2 and Purinoreceptor (P2X) 7 receptors and thus, inhibited apoptosis in human blood-derived neutrophils<sup>65</sup>. Other cathelicidin exhibit anti-apoptotic properties. Porcine cathelicidin PR-39 rescued bovine aortic endothelial cells from hypoxia-induced apoptosis by reducing caspase-3 activation via enhanced expression of inhibitor of apoptosis i.e. inhibitor of apoptosis protein-2<sup>66</sup>. On the contrary, relatively higher concentrations of LL-37 ( $\geq 20 \ \mu g/mL$  or 4  $\mu M$ ) increase apoptosis in normal human bronchial epithelial cells in a caspase-1 activation dependent manner  $^{67}$ . Through a different mechanism, LL-37 (~60  $\mu$ M) induces caspase-independent apoptosis in various colon cancer cell lines, primarily by promoting nuclear translocation of mitochondrial proteins (i.e. apoptosis inducible factor and endonuclease G)<sup>68</sup>. Thus, cathelicidin differentially regulates apoptosis, mainly dependent on the effective concentration of the peptide and the tissue environment, although specific mechanisms remain elusive.

#### 1.3.3.3. Immunomodulatory role of cathelicidin

Cathelicidin have been investigated as promising peptides that can modulate inflammatory and immune responses via their ability to interact with host membranes. Cathelicidin interact with a plethora of cell surface and intracellular receptors from a variety of immune cells (e.g., macrophages, neutrophils, dendritic cells) and non-immune cells (e.g., epithelial cells, glial
cells, endothelial cells, and fibroblasts<sup>69</sup>). For example, LL-37 activated epidermal growth factor receptor (EGFR) and p38 mitogen-activated protein kinase (MAPK) in airway epithelial cells<sup>70</sup>, insulin-like growth factor 1 receptor and extracellular signal regulated kinase (ERK) in human MCF7 breast cancer cells<sup>71</sup>, FPRL 1/ FPR 2 in human monocytes<sup>72</sup>, CXCL8 receptor cysteine-X-cysteine receptor (CXCR) 2 in human neutrophils<sup>73</sup> and an orphan G-protein coupled receptor (GPCR) called Mas-related gene X 2 (MrgX2) in human mast cells<sup>74</sup> (**Table 1.2**).

Cellular receptor	Name	Ref.
EGFR	Epidermal Growth Factor Receptor	70
IGF1R	Insulin-like Growth Factor Receptor 1	71
FPRL1/FPR2	Formyl Peptide Receptor-like 1/ Formyl Peptide Receptor 2	72
CXCR2	Cysteine-X-Cysteine Receptor 2	73
MrgX2	Mas-related gene X 2	74
P2Y11	Purinoreceptor 11	75
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase	76

**Table 1.2** Cathelicidin and potential cellular receptors.

Likewise, rat CRAMP activated a purinergic P2Y11 (GPCR) receptor on glial cells and promoted signalling through  $ERK1/2^{75}$  (**Table 1.2** and **Fig 1.6**).



**Figure 1.6.** Schematic representation of observed intrinsic receptors/signalling mechanisms of action of cathelicidin in colonic epithelium.

Chemoattraction of leukocytes has been proposed as a key immunomodulatory characteristic of cathelicidin. Cathelicidin can promote chemotaxis either through direct activation of chemokine receptors on leukocytes, or indirectly via regulating chemokine expression. Cathelicidin, LL-37, promotes *in vitro* chemotaxis of T-cells, macrophages, neutrophils, eosinophils and mast cells via interactions with the formyl peptide receptor like (FPRL) 1 receptor on their surfaces<sup>72, 77</sup> (**Table 1.2** and **Fig 1.6**). In fact, desensitization of FPRL1 in monocytes using a specific agonist for the FPRL1 receptor (Su peptide; corresponds to amino acids 563–595 of HIV envelope protein gp41) or transfection of HEK293 with FPRL1, has confirmed this chemotactic role of LL-37 and importance of FPRL1<sup>72, 78</sup>. Murine CRAMP attracted monocytes, neutrophils, macrophages and leukocytes via an FPRL1 receptor, as demonstrated by *in vitro* competitive inhibition of chemotaxis in the presence of the FPRL1 agonist MMK-1<sup>79</sup>. In addition LL-37 mediated chemotaxis of mast cells independent of FPRL1, although chemotaxis was dependent on Gi-protein-phospholipase C signalling, based on inhibitor assays<sup>80</sup> (**Table 1.2** and **Fig 1.6**).

In addition to direct activation of chemokine receptors, cathelicidin could attract leukocytes to the injury site indirectly, by promoting secretion of chemoattractants. In this regard, LL-37 induced expression of the chemokine CXCL8 (a neutrophil chemoattractant) via activation of the P2X7 receptor in human gingival fibroblasts<sup>81</sup> and via an EGFR-dependent manner in skin<sup>82</sup> and airway epithelial cells<sup>77</sup>. Cathelicidin also induced CXCL8 through mitogenactivated protein kinase kinase (MEK1/2)-p38MAPK activation in human monocytes<sup>83</sup>. Moreover, LL-37 interacted intracellularly with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in human peripheral blood-derived mononuclear cells (PBMCs) to promote expression of related neutrophil chemoattractants, including CCL-4/MIP-1β, CCL-20/MIP- $3\alpha$ , and CXCL-1/GRO- $\alpha^{76}$ . Furthermore, LL-37 stimulated HUVEC endothelial cells, murine (RAW264.7) macrophages and human peripheral blood cells to produce CCL-2 (also called monocyte chemoattractant protein (MCP)-1), a chemoattractant for monocytes, T-cells and dendritic cells<sup>84</sup>. Interestingly, LL-37 synergized with inflammatory stimuli (cytokines or pathogenic factors) in enhancing chemokine expression. For instance, LL-37 enhanced secretion of the chemokine MCP-1 and MCP-3 in the presence of pro-inflammatory IL-1ß and GM-CSF<sup>85</sup>. In response to the Gram-negative ligand LPS (TLR4 agonist), LL-37 stimulated CXCL8 secretion in bronchial epithelial cells<sup>86</sup>. During *Pseudomonas aeruginosa* infection, LL-37 enhanced neutrophil recruitment via cathepsin B, which leaked from lysosomal compartments of airway epithelial NHBE cells<sup>87</sup>.

After attracting immune cells to the site of an inflammatory or infectious insult, cathelicidin can regulate differentiation/activation of immune cells toward a particular cytokine profile, depending on cell type and inflammatory stimuli. For example, LL-37 promoted polarization of PBMCs toward a pro-inflammatory phenotype in the presence of macrophage colony-

stimulating factor, resulting in secretion of more IL-12p40 and less IL-10 upon exposure to LPS<sup>88</sup>. Similarly, LL-37 was transported to the nucleus in monocyte-derived dendritic cells to promote their maturation by inducing expression of antigen-presenting human leukocyte antigen-DR and co-stimulatory molecule CD86<sup>89</sup>. In addition, during psoriasis, LL-37 promoted its own uptake by plasmacytoid dendritic cells (pDCs) via interactions with self-DNA<sup>90</sup>. This DC induced differentiation and activation by LL-37, either alone or in combination with self-DNA, promoted expression of pro-inflammatory cytokines, including IL-12 and IFN- $\alpha^{90, 91}$ . Cathelicidin also contribute to expression and release of cytokines by non-immune cells. Human bronchial epithelial (16HBE14) cells stimulated with LL-37 secreted IL-6 in an NF- $\kappa$ B activation-dependent manner<sup>92</sup>. Likewise, LL-37, in synergy with IL-17 and IL-22, caused dose-dependent increases in IL-6 secretion by primary human keratinocytes<sup>93</sup>.

Cathelicidin also suppresses cytokine expression. LL-37 down-regulated IFN- $\gamma$ -meditated pro-inflammatory cytokine responses (TNF- $\alpha$ , IL-6 and IL-12) in human PBMCs, as well as expression of maturation markers (CD80, CD86 and MHCII) on monocytes and dendritic cells, and proliferation and class switching in murine splenic B-cells<sup>94</sup>. This suppressive role of cathelicidin has been also reported in response to bacterial pathogens. Human or murine neutrophils exposed to LL-37 had reduced secretion of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, CXCL8, and TNF- $\alpha$  in response to heat-inactivated *P. aeruginosa* or *S. aureus*<sup>95</sup>. More specifically, bacterial virulence factors seemed to modulate such cathelicidin-mediated suppression. Human or murine macrophage-like cells in the presence of LL-37 had reduced expression of TNF- $\alpha$  and nitric oxide when stimulated with bacterial LPS<sup>84, 96</sup>. Likewise, LL-37 caused dose-dependent increases in production of anti-inflammatory IL-10 and counteracted LPS-induced expression of pro-inflammatory IL-6, TNF- $\alpha$ , CXCL8, and MCP-

1 cytokines in myeloid derived dendritic cells (mDCs), pDCs, CD14+ monocytes, B-cell, and T-cells<sup>97</sup>. Ability of cathelicidin to fine-tune the immune system and thereby, act both as proand anti-inflammatory regulatory molecules, make them a unique therapeutic candidate. Mechanistically, ability of cathelicidin peptide to activate multiple receptor may provide insights into its diverse immunomodulatory properties.

# **1.4.** Pattern recognition receptors

PRRs are germline encoded receptors that recognize constitutive and conserved PAMPs from microbes or derived products. Activation of TLRs leads to production of inflammatory cytokines and co-stimulatory molecules, thus initiating innate and adaptive immune responses<sup>98</sup>. Whereas at least 10 functional TLR genes (TLR1–10) have been described in humans<sup>99, 100</sup>, mRNA genes from TLR1 to TLR9 have been identified in both small intestine and colon<sup>101, 102</sup> (**Table 1.3**).

TLR	TLR expression in normal and inflamed intestinal epithelial cells	Induction of HDPs by TLR activation in intestinal epithelial cells	Modulation of TLRs by HDPs in intestinal epithelial cells	Refs.
TLR1	RNA and protein expression	ND	ND	101, 102
TLR2	RNA and protein expression at low levels in small intestine and	ND	ND	101, 103, 104

Table 1.3 Cathelicidin interact with various TLRs in intestinal epithelium.

	abundantly in proximal			
	colon. Gene expression			
	is minimally altered in			
	IBDs.			
TLR3	Abundant RNA and	Agonists reduce	ND	101
	protein expression in	bacterial load and		
	small intestine and	inflammation by		
	colon. Gene expression	promoting murine		
	is minimally altered in	cathelicidin		
	IBDs	mCRAMP		
TLR4	Abundant RNA and	ND	Human cathelicidin	101, 103
	protein expression in		(LL-37) induce	
	colon.		TLR4 RNA and	
	Transcriptional		protein synthesis on	
	expression is increased		apical	
	in IBDs		intestinal epithelium	
			by MAPK	
			activation,	
			increasing CXCL8	
			secretion and	
			epithelial	
			antimicrobial	
			defenses against E.	
			coli	

TLR5	RNA and protein	ND	ND	101-
	expression in small and			103,
	large intestine.			105, 106
	Gene			
	expression is minimally			
	altered in IBDs			
TLR6	RNA and protein	ND	ND	101
	expression			
TLR7	ND	ND	ND	103
TLR8	RNA and protein	ND	ND	101, 102
	expression			
TLR9	RNA and protein	Tlr9 ko mice have	Human cathelicidin	101,
	expression at apical and	worsened colitis and	(LL-37) block TLR9	102,
	basolateral	reduced colonic	RNA and protein	107-109
	membranes. Gene	mCRAMP	induced by agonist	
	expression is minimally	expression	on apical intestinal	
	altered in IBDs	treatment after	epithelium	
		dextran-sodium		
		sulphate (DSS)		

In the small intestine, TLRs are mainly expressed in the crypt zone, and only weakly express in the villi<sup>103</sup>. In the large intestine, TLR expression is equally distributed across crypts and the upper part of the epithelium<sup>103</sup>. Specific for TLR types, baseline TLR4 mRNA and protein are most abundant in intestinal epithelial cells in normal colons<sup>103</sup>. Moreover, TLR3 and TLR5 mRNA and protein are abundant in normal small intestine and colon<sup>103, 108, 110</sup> (**Table 1.3**). Cytoplasmic expression of TLR protein has been described in intestinal epithelial cells, with TLR8 and TLR9 occasionally present on cell membranes<sup>111</sup>. To differentially respond to signals from the lumen or interstitium, TLRs are asymmetrically dispersed in intrinsically polarized intestinal epithelial cells, between apical and basolateral membranes. In this regard, expression of TLR2 and TLR4 are restricted to the apical membrane on differentiated enterocytes<sup>112</sup>. In contrast, TLR5 is mainly expressed at the basolateral membrane of intestinal epithelial cells, whereas TLR9 is at both apical and basolateral membranes (**Table 1.3**). This distribution of TLRs makes gut epithelial cells broad sentinels, capable of detecting both luminal and systemic pathogens.

Gut homeostasis is maintained via reduced expression of TLRs and increased expression of negative regulators of TLRs. Intestinal epithelial cells express low constitutive levels of TLR2 and TLR4 and are poorly responsive to bacterial ligands<sup>102, 113</sup>. This hyporesponsiveness to TLR ligands in the gut epithelium is attributed to a physiological mechanism to prevent exaggerated responses to microbes that normally reside at intestinal surfaces. Increasing expression of TLR inhibitory molecules is an alternative way to control TLR signalling. Some of the known intracellular negative regulators of TLRs are Toll-interacting protein (Tollip), single immunoglobulin interleukin-1 receptor-related protein (SIGIRR), interleukin-1 receptor-associated kinase (IRAK)-M, A20 and peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ )<sup>114</sup>. Of these known TLR negative regulators, Tollip, SIGIRR and PPAR- $\gamma$  regulate colonic inflammation. Tollip was originally identified as an adaptor molecule for IL-1 receptor mediated signalling in kidney epithelial (HEK293) cells, via its association with IRAK1 protein. Upon activation of TLRs or IL-1 receptor signalling, Tollip rapidly dissociates from IRAK-1, facilitating its phosphorylation by MyD88, initiating downstream NF- $\kappa$ B signalling<sup>115</sup>. Consistent with this, Tollip inhibited

LPS (TLR4) induced expression of pro-inflammatory cytokines IL-6, TNF- $\alpha$  and IFN- $\beta$  in bone marrow derived mouse macrophages<sup>116</sup> and CXCL8 in HT29 cells<sup>117</sup>. SIGIRR is expressed widely across the gut, including colonic epithelium. It inhibits the TLRinterleukin-1 receptor (IL-1R) pathway by interacting with the IL-1 receptor and tumour necrosis factor receptor-associated factor (TRAF) 6 adaptor molecule<sup>118</sup>. SIGIRR-deficient mice had increased inflammation and reduced survival (~90% dead) upon ip challenge with LPS, compared to wild type mice<sup>118</sup>. Likewise, SIGIRR deficient mice experienced increased weight loss, bleeding and higher clinical scoring in DSS-induced colitis<sup>119</sup>. The inhibitory effect of SIGIRR was not observed in bone-marrow macrophages (BMMs), suggesting that SIGIRR has a non-redundant role in regulating intestinal inflammation through its expression in epithelium<sup>119</sup>. PPAR $\gamma$  is a nuclear receptor commonly expressed by epithelial cells of colonic mucosa<sup>120, 121</sup>. Furthermore, PPARγ ligand inhibited LPS induced cyclooxygenase- 2, CXCL8 and TLR4 protein synthesis in HT29 cells<sup>122</sup>. PPAR $\gamma$  expression was reduced (by 50%) in colonic epithelium of ulcerative colitis patients, making PPARy an important therapeutic target for IBD patients. Thus, direct regulation of TLR expression and of TLR signalling by negative regulators, are mechanisms to avoid unwarranted gut inflammation under homeostasis and may be key during infection resolution.

#### **1.5.** Cathelicidin in infectious colitis

In the context of the pathogenesis of infectious colitis, cathelicidin have been proposed as novel potential therapeutic interventions. For example, ip injection of cathelicidin-WA (from *Bungarus fascia*, a venomous snake) mitigated etiologically undefined diarrhea and improved tight junctions and small intestinal epithelial morphology (i.e., increased villus height) in piglets<sup>123</sup>. In addition, lentivirus-mediated overexpression of cathelicidin prevented intestinal fibrosis in C57BL/6J mice orally challenged with *S*. Typhimurium<sup>124</sup>. Likewise, intracolonic

administration of mCRAMP prevented inflammation (reduced expression of TNF-a and tissue-specific reductions in neutrophil activity), intestinal epithelial damage and apoptosis in C57BL/6J mice infected with C. difficile<sup>125</sup>. Endogenous cathelicidin are also protective in infectious colitis. Camp-deficient mice orally infected with E. coli O157:H7 had higher microbial burden in their feces than wild type<sup>126</sup>. Whereas cathelicidin are considered broadspectrum antibacterials against both Gram positive and negative bacteria<sup>32, 127-129</sup>, relevance of such activity in vivo is controversial. These peptides have weak killing activity under physiological conditions, including high salt concentrations<sup>130</sup> or presence of sugars<sup>131</sup>. Their bacteriocidal activity is also inhibited by bacterial surface modifications such as Salmonella spp lipid A acylation<sup>132</sup>. However, given their known in vitro direct anti-bacterial activities, cathelicidin could neutralize virulent microbial factors such as LPS and DNA, via direct binding<sup>86, 133</sup>. Thus, cathelicidin could promote gut health and resolve colitis via mechanisms beyond antibacterial activity (may indirectly resolve inflammation by eliminating infectious incitants), including immunomodulation (activation and modulation of cytokines by immune cells and chemotaxis of non-hematopoietic cells) and interactions between cathelicidin and PRRs (reciprocal regulation in response to microbes/microbial determinants).

Activation of neutrophils into the gut is essential to respond to infectious agents (e.g., *S*. Typhimurium or *E. coli*.) and this could be driven by cathelicidin. In this regard, LL-37 promoted protective neutrophil response via lysosomally-leaked cathepsin B from *P. aeruginosa* infected airway epithelial cells<sup>87</sup>. Further, pre-treatment of human and murine neutrophils with LL-37 increased production of reactive oxygen species and promoted enhanced phagocytosis activity upon challenge with heat-inactivated *P. aeruginosa* or *S. aureus*<sup>95</sup>. Degranulation of mast cells is another key event in gut immunity, as mast cells release a mixture of compounds from their cytoplasmic granules, including histamine,

proteoglycans, serotonin, and serine proteases that trigger inflammatory defenses (e.g., increased permeability of the capillaries to enhance leukocytes' accessibility to infected site and thus, better odds of phagocytosing pathogens in an infected gut). LL-37 degranulated mast cells and promoted secretion of cytokines IL-2, IL-4, IL-6, and IL-31 in a GPCR- and PI3K-dependent manner<sup>134</sup>.

Cathelicidin has been also associated with promotion of wound healing and epithelial restitution through chemoattraction of endothelial and epithelial cells. LL-37 promote migration of Caco2 cells in a P2X7, EGFR, and p38MAPK activation-dependent manner<sup>135</sup>. In addition, LL-37 acted via ligand-mediated EGFR phosphorylation to induce wound healing and restitution in primary human keratinocytes<sup>136</sup>. By inducing release of CXCL8, cathelicidin may also contribute to epithelial restitution as CXCL8 stimulates cell proliferation and migration of HT29 and Caco2 cells via binding to the CXCR1 receptor<sup>137</sup>. Moreover, CXCL8 sends pro-survival signals to both fetal intestinal (H4) cells and mature Caco2 cells via CXCR-2 receptors<sup>138</sup>. In other aspects of gut healing, LL-37 induces expression of gut mucins (MUC1 and 2) in HT29 cells via MEK1/2 activation<sup>139</sup>. Reciprocally, *Muc2*-deficient mice infected with *E. histolytica* had lower expression of mCRAMP compared to wild-type C57BL/6 mice<sup>140</sup>. Thus, cathelicidin strengthens innate defenses to provide protection against pathogens as well as assist in resolving inflammation.

TLRs are key for detection of infiltrating microbes or associated virulent components and initiating synthesis of pro-inflammatory cytokines. Activation of macrophages by TLRs is a main mechanism for increased production of macrophage-derived cytokines, e.g.TNF- $\alpha$ , IL-1 and IL-6, which are characteristic of IBD<sup>141, 142</sup>. Likewise, intestinal epithelial cells express higher levels of TLR4 in IBD and it may be an additional source of pro-inflammatory

cytokines<sup>108, 143</sup>. In contrast, TLR2, TLR3, TLR5, and TLR9 were minimally altered in the intestinal epithelium of patients with IBDs<sup>106, 108</sup> (**Table 1.3**). Expression of cathelicidin can be directly modulated by TLRs or indirectly, via microbes that activate PRRs<sup>144</sup>. For instance in the corneal and conjuctival epithelial cells, stimulation of TLRs by specific agonists for TLRs1, 2, 3, 4, 5, or 6 induced LL-37 synthesis and conferred protection against bacterial infections<sup>144</sup>. As a potential negative feedback mechanism, LL-37 down-regulated mRNA expression of some TLRs (TLR5, 7, and 9) in human corneal and conjuctival epithelial cells<sup>144</sup>. Effects of TLR on LL-37 synthesis were correspondingly described in BMMs during *Mycobacterium tuberculosis* infection, in which LL-37 expression was up-regulated through bacterial ligands<sup>145</sup>. The specific importance of TLR9 in gut cathelicidin expression has been well characterized. Bacterial E. coli DNA, a natural TLR9 ligand, increased LL-37 transcription in human primary monocytes via an ERK1/2-dependent signalling pathway<sup>107</sup>. Pre-treatment with a TLR9 inhibitor, an RNA transcription inhibitor (actinomycin D), or a protein translation inhibitor (cycloheximide) inhibited LL-37 expression induced by E. coli DNA, indicating that TLR9 activation caused *de novo* RNA transcription and protein synthesis of cathelicidin<sup>107</sup>. In terms of the biological significance of TLRs modulating cathelicidin in the gut, intra-rectal administration of TLR3 agonists in mice inoculated with S. *flexneri* reduced intestinal bacterial load and mucosal inflammation by promoting expression of the murine cathelicidin mCRAMP in colonic epithelial cells<sup>146</sup>. Further involvement of TLR9 in expression of cathelicidin was reported in TLR9-deficient mice, which had worsened colitis and reduced colonic mCRAMP levels than wild-type mice after DSS treatment<sup>107</sup>. Microbial double-stranded RNA and its synthetic analogue poly(I:C), ligands for TLR3, were tissue-specific inducers of cathelicidin mRNA and protein expression in murine and human intestinal epithelial cells, through phosphatidylinositol 3-kinase-protein kinase Cζ-Sp1 pathway<sup>146</sup>. This interaction between TLR3 agonist and cathelicidin alleviated

clinical shigellosis in a murine model<sup>146</sup>. Therefore, impairment of intestinal LL-37 and TLRs interactions may be equally important in gut health.

Although TLRs can modulate expression of cathelicidin, the latter may reciprocally act on inflammatory processes by modulating expression of TLRs (**Table 1.3**). In this regard, cathelicidin in synergy with Gram-negative *S*. Typhimurium or *E. coli* or LPS increased transcription and protein synthesis of TLR4 when applied to the apical side of an intestinal epithelium<sup>109</sup>. These actions on TLR4 triggered by cathelicidin occurred mostly by activation of MAPK signalling pathways and increased CXCL8 chemokine secretion. Likewise, cathelicidin interact with a variety of TLR ligands, e.g. dsDNA (TLR9 ligand), dsRNA (TLR3) and ssRNA (TLR7 and TLR8), which depending on cell type, could either promote or inhibit pro-inflammatory cytokine expression<sup>25</sup>. For instance, LL-37 prevented TLR9 activation by oligodinucleotide (ODN) and thus, inhibited CXCL8 expression in colonic epithelial cells, whereas LL-37 promoted self-DNA mediated TLR9 activation and IFN- $\alpha$  synthesis in pDCs<sup>90, 109</sup>. Overall, role of cathelicidin in colitis and in innate defences in the gut is inherently complex and likely multifactorial. Characterization of functions and molecular mechanisms of cathelicidin during colitis could provide new insights into alternative, non-antibiotic therapeutics.

# 1.6. Hypotheses & aims

This thesis proposes two hypotheses to be tested via respective aims, as follows:

**Hypothesis 1.** *Cathelicidin senses Gram negative LPS and promotes early recruitment of neutrophils and pathogen clearance via induction of neutrophil chemotactic factor CXCL8.* 

#### **Specific aims**

# Aim 1. To elucidate the role of cathelicidin in regulating expression of neutrophil chemotactic factors in intestinal epithelial cells.

- A. To determine effect of cathelicidin in promoting chemokines involved in recruitment of neutrophils i.e. CXCL8, in intestinal epithelial cells primed by enteric pathogens and their derived virulence factors.
- B. To elucidate molecular signalling mechanisms involved in expression of CXCL8, induced by cathelicidin in intestinal epithelial cells.
- **C.** To determine biological relevance of intestinal chemotactic factors induced by cathelicidin, through evaluation of *in vitro* neutrophil activation.

# Aim 2. To determine role of cathelicidin in early resolution of infectious colitis caused by *C. rodentium*.

- A. To determine function of endogenous cathelicidin in infectious colitis in mice experimentally challenged with *C. rodentium*, in terms of promoting local microbicidal activities.
- B. To determine whether cathelicidin regulate migration of neutrophils into the inflamed colon of mice afflicted by *C. rodentium*.

C. To determine role of TLR4 and α chemokine Cxcl1/KC in cathelicidin-mediated neutrophil recruitment in mice challenged with *C. rodentium*/LPS.

**Hypothesis 2.** *Cathelicidin reduces colonic epithelial apoptosis during infectious colitis via promoting synthesis of TLR negative regulator, Tollip.* 

# **Specific aims**

# Aim 1. To elucidate role of cathelicidin in regulating expression of Tollip.

- A. To determine effect of cathelicidin in promoting Tollip expression in various *in vitro* and *ex vivo* models of colonic epithelium.
- B. To elucidate molecular signalling mechanisms involved in expression of Tollip, induced by cathelicidin in colonic epithelium.
- **C.** To determine the role of cathelicidin-induced Tollip in preventing cytokine (TNF $\alpha$  and IFN $\gamma$ ) mediated apoptosis in colonic epithelium.

# Aim 2. To determine the role of cathelicidin in preventing apoptosis in colonic

# epithelium infected by C. rodentium.

- A. To evaluate role of cathelicidin in preventing apoptosis during infectious colitis in mice experimentally challenged with *C. rodentium*.
- B. To assess the role of endogenous Tollip in preventing *C. rodentium*-induced apoptosis and validate associated molecular pathway in  $Camp^{+/+}$  and  $Camp^{-/-}$  mice.

#### **CHAPTER 2- MATERIALS AND METHODS**

### **2.1. Ethical statement**

All studies were approved by the University of Calgary Animal Care Committee (ACC-16-0092) and were conducted following Canadian Guidelines for Animal Welfare (CGAW) and principles and policies in "Guide to the Care and Use of Experimental Animals" by the Canadian Council on Animal Care. For human colonoids, 10 colonic biopsies were taken from de-identified healthy human patients, each of whom provided informed consent. All procedures were approved by the Conjoint Health Research Ethics Board at the University of Calgary (REB-18-0104).

## 2.2. Reagents

LL-37 amide trifluoroacetate salt (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and LL-37 scrambled peptide (GLKLRFEFSKIKGEFLKTPEVRFRDIKLKDNRISVQR) were purchased from Bachem (>98.6% purity, H-6224.0005) and Ana Spec (63708), respectively. Other reagents purchased were LPS from *S*. Typhimurium (437627; Calbiochem) and Alexa Fluor® 488 conjugated LPS from *S. minnesota* (L-23356; Molecular Probes). All above-mentioned reagents were re-suspended in endotoxin-free water at a final concentration of 1 µg/mL. Synthetic peptide and Alexa-488 labelled LPS were stored at -20°C whereas LPS from *S*. Typhimurium was stored at 4°C until used.

# 2.3. Colonic epithelium cell and monocyte culture

HT29 (ATCC<sup>®</sup> HTB-38<sup>™</sup>) and T84 cells, murine fibroblast L929 cells and THP-1 human monocytic cells (provided by Dr Kris Chadee, University of Calgary) were cultured in Dulbecco's Modified Eagle's Media (DMEM; Gibson, Life Technologies; for HT29, T84 and

L929 cells only) and Roswell Park Memorial Institute-1640 (RPMI-1640; THP-1 only), containing 4.5 g/L glucose with 10% fetal bovine serum (FBS; Benchmark Gemini Bio-Products), 1 mM sodium pyruvate (Gibco, Life Technologies) and 1% penicillin  $(100 \text{ Um}^{-1})$ / streptomycin (100 µg ml<sup>-1</sup>; HyClone Thermo, Fisher Scientific) in humidified environment with 5% CO<sub>2</sub>. Colonic T84 cells were grown in trans-well chambers (Corning) to form polarized monolayers with a trans-epithelial electrical resistance of >1000  $\Omega$ .cm<sup>2</sup> (monitored by an electro-voltameter; World Precision Instruments). All cells were seeded at 200,000 cells/mL. The medium was changed every 2 d and cells were passaged prior to confluence to avoid differentiation. HT29, T84 and L929 cells were used for maximum 40 passages, whereas THP-1 cells were used for a maximum of six passages.

# 2.4. Isolation of murine primary colonic epithelial cells (CECs)

Male 8-wk-old wild-type  $Camp^{+/+}$  and cathelicidin-null  $Camp^{-/-}$  C57BL/6J mice were euthanized and entire colons were aseptically removed and rinsed with cold PBS (1X). Colons were cut-open longitudinally, sectioned (2-cm long pieces) and suspended in isolation buffer containing ethylenediaminetetraacetic acid (EDTA, 5 mM; 15575020, ThermoFisher Scientific) and dithiothreitol (DTT, 0.324 M; D0632, Sigma-Aldrich) in 10 mL PBS. Isolated cells in suspension were incubated (37°C, 20 min on a rocker-shaker), passed through a cell strainer (70 µm; 10199656, VWR), washed with cold PBS (3x) and processed for cell isolation. Purity of CEC population was determined as EpCAM<sup>+</sup> (563477, BDbioscience) CD45<sup>-</sup> (103139, BDbioscience) cells using flow cytometery (~85-90%).

# 2.5. Isolation and culture of colonoids (murine and human)

Murine colonoids were developed as a 3D mini-gut model derived from isolated crypts containing intestinal stem cells and perpetuated in the presence of specialized growth-factor

enriched media<sup>147</sup>. For this, male 8-wk-old wild-type mice were euthanized as described above to collect the full-length colon. Colonic tissue was transferred to PBS (1X); fat was carefully removed and the colon cut into smaller pieces in crypt isolation buffer. This buffer contained PBS (1X), EDTA (2mM; 6381-92-6; Sigma-Aldrich), sucrose (43.4 mM; S0389; Sigma-Aldrich) and DTT (0.5 mM; 3483-12-3; Sigma-Aldrich). After removing debris, tissue was subjected to repeated centrifugation (300 *x g*, 5 min) and seeded onto Matrigel matrix (354234; Corning) in a 12-well plate containing IntestiCult<sup>TM</sup> Organoid Growth Medium (06005; Stemcell Technologies) supplemented with antibiotic-antimycotic mixture (15240062; ThermoFisher Scientific). Medium was replaced every alternate day until 10 d when tissue acquired colonoid architecture (**Fig 2.1; upper panel**).



Figure 2.1 Development of murine and human colon organoids over time.

For human colonoids, colonic crypts were isolated as described above for murine colonoids. Upon isolation, crypts were cultured on Matrigel matrix in a 24-well plate for 10 d (**Fig 2.1**; **lower panel**), until colonoid morphology was apparent. Murine and human colonoids were then treated as discussed below, and supernatants collected for quantification of CXCL8/1 using ELISA (DY208/DY453; R&D Systems).

#### 2.6. Isolation of murine bone marrow-derived macrophages

BMMs were isolated from femurs and tibias<sup>148</sup> of  $Camp^{+/+}$  and  $Camp^{-/-}$  mice. For this, bone marrow monocytes were cultured (6 d) in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 50  $\mu$ M 2-mercaptoethanol, 10 mM HEPES buffer (pH 7.4), 1% penicillin (100 U ml<sup>-1</sup>)/ streptomycin and 10% conditioned media from L929 cells (as a source of macrophage colony-stimulating factor required for macrophage lineage differentiation).

#### 2.7. Isolation of human neutrophils

Human neutrophils from healthy donors were isolated using Lympholyte®-poly solution (CL5070; Cedarlane). Fresh human blood (5 mL) was collected in a heparin-treated centrifuge tube; this anti-coagulated blood was then carefully laid on top of an equal volume of Lympholyte®-poly solution in a 15-mL tube that were centrifuged (500 x g, 35 min, RT) in a swing rotor. After centrifugation, two leukocyte bands were visible. To isolate neutrophils, the lower band of leukocytes was removed using a Pasteur pipette and put into sterile PBS (1X). Cells were washed (2x) with 10 mL of sterile PBS (300 x g, 10 min; zero de-acceleration), suspended into RPMI-1640 and kept on ice until immediate use.

#### 2.8. Bacterial culture

*S.* Typhimurium (clinical strain LT2/ATCC 700720; provided by Dr. Jeroen De Buck, University of Calgary) and *C. rodentium* (DBS-100; provided by Dr. Andre Buret, University of Calgary) were grown in Luria-Bertani (LB) Miller broth (IBI Scientific). *S.* Typhimurium and *C. rodentium* were cultured for 18 h at 37°C at 225 rpm and streaked out on a LB agar plate and a MacConkey agar plate, respectively. For experiments with *S.* Typhimurium, five colonies were taken from an agar plate and grown in LB broth (3 mL, 2 h, 37 °C at 225 rpm). The resultant culture was serially 10-fold-diluted and subjected to OD<sub>600nm</sub> determination to generate a standard curve for CFU/mL. For *C. rodentium* infection, a single colony was cultured in LB broth (5 mL overnight (O/N) at 37°) without shaking. The following day, the above bacterial culture (1 mL) was transferred into a tissue culture flask containing fresh LB broth (50 mL) and cultured for another 4 h at 37° in shaking incubator. The bacterial cells were centrifuged (4000 *x g*, 10 min, 4°C), washed with PBS (2x), and suspended at a concentration of ~5 x  $10^8$  CFU/mL or ~25 x  $10^8$  CFU/mL as noted in specific experiments.

# 2.9. Host cell stimulation

Colonic epithelial cells or macrophages were treated with LPS and LL-37, either alone or in combination, for variable intervals and at variable concentrations as noted in respective figures. HT29 cells were stimulated with *S*. Typhimurium, at a multiplicity of infection (MoI) of 10:1 (~ 2 x 10<sup>7</sup> CFU; OD<sub>600</sub> (1) = ~ 0.8 x 10<sup>8</sup> CFU/mL), heat-inactivated *S*. Typhimurium (2 x 10<sup>8</sup> CFU equivalent; 15 min at 70°C), flagellin (1 µg/mL; SRP8029; Sigma-Aldrich), ODN (1 µg/mL; tlrl-2395; Invivogen) or a combination of TNF- $\alpha$  (variable) and IFN- $\gamma$  (300 IU/mL) as indicated in figures. These treatments were either combined or preceded by synthetic LL-37 or scrambled LL-37 (sLL-37) peptide, as indicated in respective figures. Cytotoxicity induced by *S*. Typhimurium (~ 2 x 10<sup>7</sup> CFU), variable concentrations of LPS (10 ng/mL to 5 µg/mL) and LL-37 (1 µg/mL to 30 µg/mL) or cytokine cocktail (TNF- $\alpha$  and IFN- $\gamma$ ) were determined using a Pierce<sup>TM</sup> Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (88953; ThermoFisher Scientific).

THP-1 human monocytic cells were differentiated with PMA (20 ng/mL; P8139; Sigma-Aldrich) for 3 d<sup>149</sup>, followed by simultaneous treatment with LPS (1  $\mu$ g/mL) and LL-37 (10  $\mu$ g/mL), either alone or in combination for 4 h. BMMs cultured in RPMI without FBS and

antibiotics were challenged with LPS (1  $\mu$ g/mL) either for 24 or 1 h (for ELISA or immunoblotting, respectively).

# 2.10. Use of pharmacological inhibitors for studying signalling pathways

Potentially involved signalling pathways were investigated by pharmacological blockade. Targets and inhibitors included human TLR4 using a competitive inhibitor LPS isolated from Rhodobacter sphaeroides (LPS-RS, tlrl-prslps; Invivogen) and polymyxin B sulfate salt (1405-20-5; Sigma-Aldrich), human EGFR 2/ ErbB2 (10 µM; TAK 165, 366017-09-6; Tocris Bioscience), FPRL1 (WRW4, 2262; Tocris Bioscience), matrix metalloproteinases (10 µM; MMP; GM6001, 2983; Tocris Bioscience), P2X7 (10 µM; A740003, 3701; Tocris Bioscience), EGFR-kinase (10 µM; AG1478 hydrochloride, 1276; Tocris Bioscience), Sarcoma kinase (Src)-kinases (1 µM; PP1, Calbiochem) and MEK (also known as MAPKK) 1/2 (20 µM; PD98059, 9900; Cell Signaling Technology and U0126, 1144; Tocris Bioscience), p38 MAPK (10 µM; SB203580, 1202; Tocris Bioscience), ERK (10 µM; FR180204, 3706; Tocris Bioscience) and NF- $\kappa$ B activating kinase i.e. I $\kappa$ B kinase  $\beta$  (10  $\mu$ M; PS-1145; Cayman Chemical). Endocytic processes were investigated by inhibiting endocytosis (10 µM; D15, 2334; Tocris Bioscience), actin polymerization (2 µM; Cytochalasin D, C8273; Sigma-Aldrich), and lipid raft formation (250 ng/mL, Mevinolin, M2147, HMG-CoA reductase inhibitor; Sigma-Aldrich and 500 μM, methyl-β-cyclodextrin, 332615, cholesterol solubilizing agent; Sigma-Aldrich). Cycloheximide (1 µM, 2112; Cell Signaling Technology) and actinomycin D (1 µM, 15021; Cell Signaling Technology) were utilized to assess gene translation and transcription, respectively. Inhibitors in fresh serumfree media were added to cells 1 h prior to respective treatment. As a control, cells received equivalent doses of DMSO in serum-free medium.

#### 2.11. Quantification of secreted cytokines

Protein secretions for CXCL8/1, LL-37, TNF-α or IL-6 were determined in supernatants upon respective treatments as discussed above, using ELISA kits for CXCL8 (DY208; R&D Systems) and/or LL-37 (only HT29 cells) (HK321; Hycult Biotechnology) or Cxcl1 in murine colonoid supernatant. THP-1 cell supernatants were evaluated for CXCL8 and TNF-α (DY210; R&D Systems) whereas mouse TNF-α (DY410; R&D Systems) and IL-6 (DY406; R&D Systems) were determined in BMMs supernatants. In these ELISAs, samples were assessed at 450 nm in a microplate reader and subtracted from reading at 540 nm to account for wavelength correction. Data were represented as absolute values (pg/mL). Data from human colonoids were represented as fold increase in CXCL8 secretion, to account for interpatient variability.

# 2.12. Protein identification by Western blotting

Cells grown in six-well plates and treated as discussed above, were scraped using ice-cold PBS (1X) with protease inhibitor cocktail (87786; ThermoFisher Scientific). Upon centrifugation (1500 *x g*, 5 min), pellets were re-suspended in denaturing cell extraction buffer (FNN0091; Thermo Fischer Scientific) and proteins were isolated. Protein quantification was done using Pierce BCA Protein Assay Kit (23225; Thermo Fisher Scientific), followed by resolution using appropriate SDS-PAGE concentration. Proteins were transferred to methanol-activated polyvinylidene difluoride (1620177; Bio-Rad) membrane. The membrane was blocked for 2 h either with 5% bovine serum albumin (9048-46-8; Amresco) or 5% non-fat dry milk (for non-phophorylated proteins) dissolved in Trisbuffered saline plus 0.1% Tween 20 (TBST) solution. Resolved proteins were blotted using specific primary antibodies to detect phospho-EGFR-Tyr1068 (2234; Cell Signaling Technology), EGFR (4267; Cell Signaling Technology), phospho-p38 MAPK-

Thr180/Tyr182 (4511; Cell Signaling Technology), p38 MAPK (9212; Cell Signaling Technology), phospho-ERK1/2 (4370; Cell Signaling Technology), phospho-NF-κB p65-Ser536, human Tollip (ab187198; Abcam and 4748; Cell Signaling Technology), mouse Tollip (97632; NovusBio), cleaved caspase-3 (9661; Cell Signaling Technology), cleaved PARP (9542; New England Biolabs), phospho-AGO 2-Ser393 (ab215746; Abcam) and human GAPDH-6C5 (1001; Calbiochem). Horseradish-peroxidase-conjugate (HRP) goat anti-mouse IgG (H+L) (115-035-146; Jackson ImmunoResearch) or HRP goat anti-rabbit IgG (H+L) (115-035-144; Jackson ImmunoResearch) were used as secondary antibodies and developed using the Clarity Western ECL Detection System (BioRad). Image capture and densitometric analyses were performed with ChemiDoc MP Imaging system and ImageLab 4.0.1 software (BioRad), respectively. Normalization was done with reference to either GAPDH (housekeeping protein) or respective total protein (EGFR/p38MAPK). Results were reported as mean fold change of target expression in stimulated groups, compared to an unstimulated control group.

#### 2.13. mRNA quantification of innate factors

RNA was extracted from cells using an RNA extraction reagent (Ribozol, Amresco). Relative mRNA synthesis was quantified by quantitative real time polymerase chain reaction (qPCR). For these studies, RNA samples (1 μg) were reverse transcribed using a cDNA synthesis kit (qScript, Quantbio). RNA and DNA quality and quantity were assessed with a spectrophotometer (NanoVue, GE Healthcare Bio-Sciences Corp). Absence of contaminating genomic DNA from RNA preparations was checked using a minus-RT control (i.e., sample with all RT-PCR reagents except RT). qPCR was performed using a 96-well real time PCR system (CFX-96, BioRad). Each reaction mixture contained 100 ng of cDNA, 1X SYBR Green Supermix (SsoAdvanced Universal, BioRad) and 0.5 μM of each specific primer, in a

final volume of 10 µL. Pre-designed primers (RT<sup>2</sup> qPCR Primer Assay, Qiagen) specific for human CXCL8 (PPH00568A; NM 000584.3), human Tollip (PPH05844C; NM 019009), human *IFN-γ* (PPH00380C; NM\_000619), human *TNF-α* (PPH00341F; NM\_000594), human *IL-1β* (PPH00171C; NM\_000576), human *GAPDH* (PPH00150F; NM\_002046.5), mouse Tollip (PPM06269E; NM\_023764), mouse Gapdh (PPM02946E; NM\_008084) with verified specificity and efficiency (> 95%) to ensure amplification of a single product of the correct size, as indicated in MIQE guidelines<sup>150</sup>. GAPDH, along with two other housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase 1 and phosphoglycerate kinase 1 were optimized, and found to be invariable across treatment groups. In agreement, GAPDH has been reported to be the most desirable house-keeping gene for HT29 cells<sup>151</sup>. Therefore, values of target mRNA were corrected relative to the normaliser GAPDH. Reaction mixtures were incubated for 95°C for 5 min, followed by denaturation for 5 s at 95°C and combined annealing/extension for 10 s at 60°C (total of 40 cycles). Negative controls for cDNA synthesis and PCR procedures were included in all cases. Data were analysed using the  $2^{-\Delta\Delta CT}$ methods and results reported as mean fold change of target transcription levels in stimulated groups versus an untreated control group.

For processed form of precursor micro RNA-31 (miR-31) quantification, total RNA was subjected to poly A tailing prior to cDNA synthesis (95107; QuantaBio). miR-31 abundance was assessed using microRNA qPCR assay primer for miR-31-5p (MystiCq, MIRAP00090; Sigma-Aldrich) and RNU6-1 (MIRCP00001; Sigma Aldrich). Target miRNA values were corrected relative to the normaliser, RNU6-1.

#### 2.14. CXCL8 mRNA stability determination

Stability of *CXCL8* mRNA in HT29 cells was determined by an actinomycin D chase assay. Cells were washed with PBS (1X) after challenge with LPS  $\pm$  LL-37 (for 2 h) and, actinomycin D alone or in combination with p38MAPK inhibitor (SB203580) was added for the indicated intervals. RNA was isolated (Ribozol, RNA extraction), as discussed above. Following cDNA synthesis and subsequent qPCR, data were represented as percentage *CXCL8* mRNA remaining compared to actinomycin D only treatment as control.

# 2.15. Colonic epithelial LPS uptake assay

HT29 cells grown in eight-well chambers (Thermo Fischer Scientific) were treated with Alexa488 fluorescent conjugated-LPS and/or LL-37. Following incubation for a specified interval (see Chapter III for details), cells were fixed with 4% paraformaldehyde (PFA) in dark (15 min; RT) and rinsed in ice cold PBS with 0.05% Tween-20 (PBS-Tw, 3x, pH 7.2). Cells were counterstained for nuclei with 4', 6-diamidino-2- phenylindole (DAPI; 1: 1,000) (30 min; RT), rinsed with PBS-Tw (3x) and mounted with FluorSave reagent (Calbiochem, EMB Millipore). Slides were examined using wide-field immunofluorescence microscope (IX710lympus). This was followed by qualitative and quantitative assessment of Alexa488-LPS uptake by colonic cells. For analysis, 8-10 cells per replicate were randomly selected and fluorescence intensity was quantified using ImageJ software (National Institute of Health, USA). The intensity was averaged over three replicates per experiment (total of three independent experiments, unless mentioned otherwise) and represented as mean fluorescent intensity (MFI) per cell.

#### 2.16. Assessing TLR4 location in colonic epithelium

HT29 cells (treated with LPS  $\pm$  LL-37 or not) were either permeabilized (for intracellular TLR4) or not (surface TLR4), followed by analysis using immunofluorescence or flow cytometery. For immunofluorescence, cells were fixed as discussed above and permeabilized (or not) using 0.1% Triton X-100 (T8787; Sigma-Aldrich). Cells were then incubated with anti-TLR4 primary antibody (ab89455; Abcam) (1:100, O/N, 4°C) followed by cyanine 3conjugated donkey anti-mouse IgG (H+L) (715-165-150; Jackson ImmunoResearch) secondary antibody (1:200, 1 h, RT). Cells were counterstained for nuclei with DAPI (1:1000, 30 min, RT) and/or Alexa647-conjugated wheat germ agglutinin (WGA, W32466; ThermoFisher Scientific) (1:500, 30 min, RT), which binds membrane expressing N-acetyl-D-glucosamine and sialic acid. Slides were examined using wide-field immunofluorescence microscope (IX710lympus). Data were reported as MFI for three independent experiments. For flow cytometeric analysis, HT29 cells permeabilized (or not) were incubated either with PE labelled anti-TLR4-CD284 (12-9917-41; eBioscience) or PE labelled isotype mouse IgG (12-4724-41; eBioscience) (0.5 µg/mL, 30 min, 4°C). Cells were then analysed using flow machine (BD<sup>TM</sup> LSR II; BD-Bioscience). Data were obtained as MFI and represented as TLR4 expression normalized to surface expression.

#### 2.17. Plasmid transfection

A short hairpin ShTLR4/ShTollip/ShLL-37 pGFP-V-RS or mir31 pCMV-MIR plasmid vector or non-effective scrambled ShRNA construct (sham) (TG320555 and TG314213; Origene) were transfected into confluent HT29 cells (HT29ShTLR4, HT29ShTollip, HT29ShLL-37 and HT29MIR31, respectively) using Cell Line Nucleofector® Kit V (VCA-1003; Lonza). Transfected cells were selected for puromycin (30 µg/mL for ShTLR4 and ShLL-37 and 5 µg/mL for ShTollip) or neomycin (800 µg/mL for MIR31) resistance.

Knockdown efficiency was ~90% for TLR4, Tollip and LL-37, whereas overexpression was 300% for mir31, as assessed through relative intensity quantification of TLR4 and Tollip protein bands, LL-37 ELISA (HK321; Hycult Biotechnology) or qPCR for miR-31 levels, respectively. Cells were maintained by constantly culturing them in DMEM media supplemented with either puromycin (15 μg/mL for ShTLR4 and ShLL-37 and 2.5 μg/mL for ShTollip) or neomycin (400 μg/mL for MIR31).

# 2.18. Luciferase promoter-based assay

A pGL3 basic plasmid containing 174bp CXCL8 promoter construct (-166 to +8 relative to transcriptional start site) upstream of a firefly luciferase gene and a pRL null plasmid constitutively expressing renilla luciferase gene were used (provided by Dr. David Proud, University of Calgary). The CXCL8 promoter construct contained either intact (174 bp full length; FL) or individually mutated sites for NF- $\kappa$ B, AP-1 and Nuclear factor for IL-6 expression (NFIL-6) (site-directed mutagenesis; denoted as mNF- $\kappa$ B, mAP-1 and mNFIL-6, respectively) (**Fig 2.2**).



**Figure 2.2** Gene map for various CXCL8 luciferase promoter constructs and their cloning into pGL3 basic plasmid.

HT29 cells were co-transfected with construct (1 μg) and pRL (0.1 μg) in DMEM media (FBS and antibiotic free) using TransIT transfection reagent (MIR5405; Mirus Bio LLC). pRL null plasmid was used as transfection control. Recombinant human interleukin-1β (IL-1β; 10 ng/mL, 8900; Cell Signaling Technology) was used as a positive control for CXCL8 promoter assay. Results were quantified using a dual luciferase reporter assay (PR-E1910; Promega). Data were obtained as fold change in relative light units (RLU) of firefly luciferase activity with respect to control group, for three independent experiments. For miR-31, a pMIR-31- reporter plasmid (provided by Mien-Chie Hung, Addgene 71871) was transfected as described above. Data were calculated as inverse of firefly luciferase activity, normalized to control and reported as '% miR-31 expression'.

# 2.19. Human neutrophil calcium flux assay

Neutrophils were isolated from blood of healthy donors, as discussed above. For calcium flux assay, neutrophils were incubated with Fluo4-no wash calcium dye (Fluo4 NW, F1242; Invitrogen) (1 mL; 45 min, RT), washed with PBS and re-suspended in calcium-magnesium containing HBSS. Neutrophils (1 x 10<sup>6</sup>) were transferred into a cuvette and stimulated with supernatants (100 µL) harvested from either untreated (control; serum free DMEM media) or LPS and/or LL-37 treated HT29 cells. To confirm the role of CXCL8, either supernatants from LPS-LL37 treated HT29 cells were blocked (or not) with anti-CXCL8 antibody (1 µg/mL, MAB208; R&D Systems) or mouse IgG1 isotype control (1 µg/mL, 5415S; Cell Signaling Technology) (1 h, 4°C) or with CXCR1/2 inhibitor SCH 527123 (20 µM, A3802; APExBIO), prior to stimulation of neutrophils. Recombinant human CXCL8 (rCXCL8; 200 ng/mL, 208-IL-010; R&D Systems) was used as positive control. For calcium flux assay, calcium signals were monitored at an excitation wavelength of 480 nm and an emission wavelength of 530 nm, recorded with an Aminco Bowan Series II fluorimeter and the AB2

software (Thermo Fisher Scientific). Data were expressed as a percentage of the emission fluorescence normalized to maximum fluorescence caused by  $2.5 \mu$ M calcium ionophore A23187. All data recorded were within the range of fluorescence obtained with calcium ionophore A23187.

#### 2.20. Neutrophil-extracellular-traps (NETs) quantification

To assess the role of CXCL8 secreted by colonic epithelial cells in NETosis, human neutrophils were isolated as discussed above and seeded in eight-well chambers at  $1 \ge 10^6$ cells/well. Neutrophils were stimulated for 4 h with supernatants (500 µL) harvested from either untreated (control; serum free DMEM media) or LPS and/or LL-37 treated HT29 cells. Neutrophils were fixed in 4% PFA (15 min, RT) and rinsed in ice cold PBS-Tw (pH 7.2), followed by blocking in 10% donkey serum (017-000-021; Jackson ImmunoResearch), 1% bovine serum albumin and 0.3 M glycine in PBS-Tw (1 h, RT). After rinsing with ice cold PBS-Tw, neutrophils were incubated with primary anti-neutrophil elastase antibody (5 µg/mL; MAB91671; R&D Systems) diluted in PBS (O/N, 4°) and then, incubated with secondary Alexa Fluor 594- conjugated donkey anti-mouse IgG (H+L) (711-605-152; Jackson ImmunoResearch) antibodies diluted 1:1,000 in 1% BSA in PBS-Tw (RT; 1 h). Following rinsing in PBS-Tw, neutrophils were counterstained with DAPI (62247; ThermoFisher Scientific) (1:1,000) and mounted with FluorSave (345789; Calbiochem). Slides were examined using a wide-field immunofluorescence microscope (IX71; Olympus). For NETs quantification, MFI for five fields of views per replicate, was calculated using ImageJ software. The average fluorescence intensity was then determined per experiment (three replicates each, i.e., 15 fields of view/experiment) and obtained as a single numerical value. Data are numerical values obtained from three independent experiments.

#### 2.21. Mice experiments

Male 8-wk-old wild-type *Camp*<sup>+/+</sup> and cathelicidin-null *Camp*<sup>-/-</sup> C57BL/6 mice (B6.129X1-*Camp*<sup>tm1Rlg</sup>/J; The Jackson Laboratory) were housed in a pathogen-free environment (University of Calgary). These *Camp*<sup>-/-</sup> mice had no "off target" genetic defects accompanying mutation in the CRAMP locus<sup>152</sup>.

# 2.22. Intra-peritoneal injection of LPS

*Camp*<sup>-/-</sup> mice were injected ip with LPS (437627; Calbiochem) and/or LL-37 (H-6224.0005; Bachem) (both at 1  $\mu$ g/g), diluted in pyrogen-free saline (*n*= 2-5 mice/per group). Control mice were sham-injected with the saline vector. Mice were euthanized 3 h post challenge using 5% isoflurane anaesthesia, followed by cervical dislocation. Sections of the distal colon were sampled, weighed, suspended in PBS (50 mg/mL; Gibco, Life Technologies; 1X) with protease inhibitor cocktail (87786; ThermoFisher Scientific), homogenized using tissue homogenizer and stored at <sup>-</sup>80°C for subsequent ELISA quantification for Cxcl1, as explained below.

# 2.23. C. rodentium infection of mice

A *C. rodentium* DBS-100 strain was used to experimentally induce colitis in mice, as described<sup>153</sup>. Each mouse ( $Camp^{+/+}$  or  $Camp^{-/-}$ ) was inoculated orally with 200 uL (~1 x 10<sup>8</sup> CFU or ~5 x 10<sup>8</sup> CFU, specific to an experiment); a dose sufficient for establishing infection in C57BL/6 mice<sup>154</sup>. Control mice were sham-inoculated with 200 uL of PBS (1X). Mice were euthanized on day 7 post *C. rodentium* infection, followed by excision of colonic tissues.

#### 2.24. Quantification of secreted proteins

Sections of distal colon were weighed and suspended in PBS containing protease inhibitors (87786; ThermoFisher Scientific) (50 mg tissue wet-weight/mL)<sup>155</sup> to conduct CXCL1 (DY453; R&D Systems) and lipocalin-2 (DY1857; R&D Systems) quantitative ELISAs. Data are presented as absolute values normalized to tissue wet weight.

#### 2.25. Protein determination in colonic tissue by Western blotting

Distal colon was sampled, weighed and suspended in denaturation cell extraction buffer containing protease inhibitor cocktail (50 mg/mL). Protein blotting for phospho-EGFR-Tyr1068, phospho-NF-κB p65-Ser536 and human GAPDH-6C5 was performed, as discussed above.

#### 2.26. Myeloperoxidase (MPO) activity assessment

Tissues were weighed, suspended in hexadecyltrimethylammonium bromide buffer (50 mg/mL), homogenized using tissue homogenizer and centrifuged (1500 *x g*, 4 min, 4°C). Tissue supernatant (7  $\mu$ L) was mixed with a O-dianisidine solution (200  $\mu$ L), containing O-dianisidine (0.5mM) and hydrogen peroxide (1%) in potassium phosphate buffer. Optical absorbance density was recorded at 410 nm and data were represented as MPO activity in units (U)/g<sup>156</sup>.

#### 2.27. Histological assessment and immunofluorescence

Fresh colonic tissues (<1 cm) were fixed in 10% neutral buffered formalin for 3 h, transferred to ethanol 100% at 4°C and embedded in paraffin blocks for histological staining and immunofluorescence studies. Paraffin sections (5  $\mu$ m) were deparaffinized by xylene substitute (Neo-Clear 65351-85 Millipore), followed by decreasing concentrations of ethanol.

The protocol of Hematoxylin & Eosin (H&E) staining for microscopic pathology was performed as described<sup>140</sup>. For immunofluorescence, after deparaffinization, slides were washed under running tap water (5 min) and rinsed in ice cold PBS-Tw (pH 7.2), followed by blocking in PBS-Tw containing 10% donkey serum (017-000-021; Jackson ImmunoResearch), 1% bovine serum albumin (9048-46-8; Amresco) and 0.3 M glycine (1 h, RT). After rinsing with ice cold PBS-Tw, slides were incubated with primary Alexa647 tagged-anti-lymphocyte antigen 6 complex locus G6D (Ly6G) antibody (5 µg/mL; MAB91671; R&D Systems) or anti-mouse Tollip antibody (1:100) diluted in PBS (16 h, 4°C). Slides were rinsed with PBS-Tw, counterstained with DAPI (62247; ThermoFisher Scientific) (1:1,000) and mounted with FluorSave (345789; Calbiochem). Slides were examined using wide-field immunofluorescence microscope (IX71; Olympus). Integrated fluorescence intensity per mouse was calculated using ImageJ (1.50i) software in five randomly selected fields of view and data were reported as MFI from n= 4 mice. Apoptosis in colons was assessed by TUNEL staining (ApopTag Red In Situ Apoptosis Detection, 57165; Sigma-Aldrich).

# 2.28. Quantification of secreted proteins by multiplex cytokine array

CECs were isolated as discussed above. Secretion of chemokines was assessed in CECs by multiplex bead-based assay (Chemokine Discovery MD31) by Eve Technologies (Calgary, AB, Canada). Data for multiplex chemokine assay were normalized to total amount of protein (pg/mL).

#### 2.29. Bacterial quantification in feces, liver and spleen

To measure CFU/g of *C. rodentium* in liver and spleen, these organs were homogenized in PBS (50 mg/mL) and lysates plated on MacConkey agar plates at various dilutions for

quantification of *C. rodentium* colonies. To determine fecal shedding of *C. rodentium*, fresh fecal pellets (one per mouse) were obtained aseptically from infected mice at 3, 5 and 7 d pi, and cultured on MacConkey agar plates at various dilutions. Bacterial colonies were counted, and data reported as fold increase in CFU/g normalized to *Camp*<sup>+/+</sup> infected mice. qPCR validation was done using *C. rodentium* specific primers against *espB* (extracellularly secreted protein B) gene (forward primer: 5'-ATGCCGCAGATGAGACAGTTG-3' and reverse primer: 5'-CGTCAGCAGCCTTTTCAGCTA-3')<sup>157</sup>.

# 2.30. Statistical analyses

Graphs represent normally/Gaussian distributed (parametric) results represented as means and bars represent standard errors of the mean (SEM) from a minimum of three independent experiments, with each experiment repeated in triplicates, unless otherwise mentioned. Normality was assessed using Shapiro-Wilk test. All comparisons were performed using either two-sided, unpaired Student's *t*-tests or one-way analysis of variance (ANOVA) with Bonferroni correction for multiple group comparisons. A *p* value was assigned to each group with reference to control group, unless shown specifically on the graph. A *p* value of <0.05 was considered significant. A *p* value was given only to group comparisons with significant differences. All statistical analyses were performed with Graph Pad Prism software.

#### **CHAPTER 3- RESULTS**

Study 1. Cathelicidin triggered neutrophil chemotactic factor CXCL8 synthesis in colonic epithelium in response to Gram negative LPS and promoted early recruitment of neutrophils

# **3.1.** Cathelicidin synergized with *S*. Typhimurium or LPS to stimulate human CXCL8 production.

LPS containing Gram-negative bacteria, such as *S*. Typhimurium and the murine-specific pathogen *C. rodentium*, induce CXCL8 and CXCL1 synthesis by the colonic epithelium<sup>158, 159</sup>. The only cathelicidin in humans, LL-37, induces CXCL8 synthesis in airway and corneal epithelial cells<sup>70, 160</sup>. However, whether cathelicidin assists LPS containing Gram-negative bacteria in CXCL8/1 secretion by colonic epithelium is unknown. We assessed CXCL1 secretions in CEC extracts from  $Camp^{+/+}$  and  $Camp^{-/-}$  mice infected with *C. rodentium* at the peak of infection (7 d pi)<sup>161</sup>. CEC secreted CXCL1 concentrations were 2-4-fold higher in infected  $Camp^{+/+}$  than  $Camp^{-/-}$  mice (**Fig 3.1A**). Likewise, secretion of a related chemokine, CCL3, was higher in CEC isolated from *C. rodentium* infected  $Camp^{+/+}$  mice compared to the corresponding  $Camp^{-/-}$  CEC (**Fig 3.1B**). In contrast, secretions of chemokines CXCL9 and CXCL10 were increased in *C. rodentium* infected  $Camp^{-/-}$  CEC (**Fig 3.1(C-D**)); whereas kinetics of other chemokines (CCL5 and CXCL2) were not significantly different between *C. rodentium* infected  $Camp^{+/+}$  and  $Camp^{-/-}$  CEC (**Fig 3.1, E-F**)).



Figure 3.1. Chemokine production in CECs isolated from *C. rodentium* infected  $Camp^{+/+}$  and  $Camp^{-/-}$  mice.  $Camp^{+/+}$  and  $Camp^{-/-}$  mice were infected with *C. rodentium* (1 x 10<sup>8</sup> CFU in 200 µL of PBS) for 7 d. Chemokine production of CXCL1 (A), CCL3 (B) CXCL9 (C), CXCL10 (D), CCL5 (E) and CXCL2 (F) were determined in isolated CECs, using multiplex bead-based assay. Data are mean  $\pm$  SEM (n= 3-6/group). P < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-tests for two groups) was considered significant.

In regard to colitis hallmarks, both groups of infected mice had comparable reductions in cecum weight (**Fig 3.2A**); however, only infected  $Camp^{+/+}$  mice had increased crypt length (**Fig 3.2B**) and decreased colon length (**Fig 3.2C**).



Figure 3.2. Histopathological determination of colitis in  $Camp^{+/+}$  and  $Camp^{-/-}$  mice challenged by *C. rodentium*. (A-C) C57BL/6  $Camp^{+/+}$  and  $Camp^{-/-}$  mice were orally infected with *C. rodentium* (1 x 10<sup>8</sup> CFU in 200 µL of PBS) for 7 d. (A) Representative pictures for appearance of cecum in both  $Camp^{+/+}$  and  $Camp^{-/-}$  mice. Bar graph representing total cecum weight in PBS control and infected mice. (B) H&E staining of mice colonic tissues, and histograms depicting crypt length. Black arrows in H&E pictures denote crypt length. (C) Histograms representing colon length in control as well as infected mice. Data are means ± SEM (n=4/group). *P* < 0.05 (One-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) was considered significant.

Next, we wanted to determine whether cathelicidin induced CXCL1 synthesis in the presence of the Gram-negative bacterial factor LPS. Only the combination of LL-37 and LPS (neither LL-37 alone nor LPS + sLL-37 peptide) enhanced CXCL1 secretion in  $Camp^{-/-}$  mice when administered ip for 3 h (**Fig 3.3A**). Results were similar to an *ex-vivo* model of murine colonoids developed from  $Camp^{+/+}$  mice (4 h) (**Fig 3.3B**).

To examine *in vitro* cooperation between cathelicidin and Gram-negative bacteria, HT29 cells were rendered cathelicidin deficient via knock-down (ShLL-37). These cells had reduced secretion of CXCL8 when exposed to *S*. Typhimurium at early (2 h) and later (8 h) time points compared to sham-transfected cells (**Fig 3.3C**), with comparable cytotoxic side
effects (**Fig 3.3D**). To determine if a live bacterium was required for CXCL8 synthesis, HT29 cells were challenged with heat-killed *S*. Typhimurium; there were synergistic increases in CXCL8 secretion with heat-killed *S*. Typhimurium and LL-37 (**Fig 3.3E**).



Figure 3.3. CXCL1/CXCL8 synthesis in colonic epithelium stimulated with cathelicidin and LPS or S. Typhimurium infection. (A-B) CXCL1 protein expression determined by ELISAs in homogenized colons of Camp<sup>-/-</sup> mice (n=3-5 mice/per group) (A) or colonoids from  $Camp^{+/+}$  mice (**B**) treated with LPS (1 µg/g or 1 µg/mL), LL-37 (1 µg/g or 10 µg/mL) or scrambled-sequence peptide sLL-37 (10 µg/mL; only in mouse colonoids), either alone or in combination (ip for 3 h in (A) and ex vivo for 4 h in (B)). (C-E) HT29 cells either transfected with sham plasmid/knocked-down for LL-37 (ShLL-37) or untransfected were challenged with S. Typhimurium (MoI 10:1;  $2 \times 10^7$  CFU) (C-D) or heat-killed S. Typhimurium (MoI 100:1; 2 x 10<sup>8</sup> CFU) (E) for variable time points (up to 8 h). Bar graphs represent LL-37 secretions (1 fold ~ 250 pg/mL) (C; upper graph) and CXCL8 secretions (C; lower graph, and E) in cell supernatants as assessed by ELISA. (D) Cellular toxicity was evaluated by LDH assays. Data were represented as percentage increase in LDH activity normalized to control. (C) Graph is representative of three independent experiments. Data are mean  $\pm$  SEM (n=3 independent experiments done in triplicate, unless mentioned otherwise in respective)sub-figure). P < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's t-test for two groups) was considered significant. N.D= 'not detected'.

To further validate LPS and LL-37 synergism *in vitro*, we used two separate models of colonic epithelia: HT29 and T84, and human colonoids. There was enhanced CXCL8 secretion in the presence of LPS and LL-37 (but not sLL-37) in HT29 cells at both earlier and later time points (4 h (**Fig 3.4A**) and 16 h (**Fig 3.4B**)), in T84 cells (only apical secretion was observed; **Fig 3.4C**), as well as in colonoids derived from healthy human colon biopsies (**Fig 3.4D**). Further, to evaluate roles of other TLR ligands in synergizing with cathelicidin, we challenged HT29 cells either with agonists for TLR9 (ODN) or TLR5 (flagellin). LL-37 reduced ODN mediated CXCL8 secretion in HT29 cells but, no effect (synergistic or antagonistic) was observed with the TLR5 agonist flagellin (**Fig 3.4B**).



Figure 3.4. CXCL8 synthesis in colonic epithelium, and CXCL8 and TNF- $\alpha$  synthesis in THP-1 monocytes stimulated with cathelicidin and LPS. (A-E) CXCL8 and TNF- $\alpha$  (E) were determined by ELISA in cell supernatants from HT29 cells (A-B), T84 cells (C), human colonoids (D) and human monocyte THP-1 cells (E). Challenge consisted of LPS (1 µg/mL) (A-E), flagellin (Flag; 1 µg/mL) or ODN (1 µg/mL) (B) and LL-37 (10 µg/mL) (A-E), either alone or in combination for 4 h (16 h in B). Data are mean ± SEM (*n*= 3 independent experiments done in triplicate, unless mentioned otherwise). *P* < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) was considered significant.

In contrast to colonic epithelium, antagonistic combinatorial effects between cathelicidin and LPS have been reported in leukocytes. For instance, LL-37 reduces LPS-mediated CXCL8 and TNF- $\alpha$  cytokine secretion in human (THP-1) monocytes<sup>162</sup>. To determine cell specificity of cathelicidin in inflammatory responses, PMA differentiated THP-1 cells were used. Although we did not observe synergistic or antagonistic effects of exogenous LL-37 on LPS induced CXCL8 secretion, LL-37 did reduce LPS-induced TNF- $\alpha$  production at 4 h (**Fig 3.4E**). Concentrations of LL-37 (10 µg/mL) and LPS (1 µg/mL) (**Figs 3.5(A-B) and 3.5(C-D**)), and the time-point (4 h) examined (**Fig 3.5E**) were based on our studies of CXCL8 kinetics. Next, we wanted to assess the role of endogenous cathelicidin in regulating pro-inflammatory cytokines in an *ex vivo* model of mononuclear cells i.e., BMMs. Unlike THP-1 cells, *Camp*<sup>+/+</sup> BMMs produced higher TNF- $\alpha$  and IL-6 upon LPS challenge than *Camp*<sup>-/-</sup> BMMs (**Fig 3.5F**). Thus, LL-37 synergises with Gram-negative pathogens or LPS in the colonic epithelium to augment synthesis of chemokines CXCL8 and CXCL1.



Figure 3.5. Dose and time curves for CXCL8 synthesis in colonic epithelial cells in presence of LPS and/or LL-37 and cytokine secretion by BMMs. (A-E) HT29 cells were challenged with variable concentrations of LPS alone (A), LL-37 alone (B), constant LPS (1  $\mu$ g/mL) and variable concentrations of LL-37 (C), constant LL-37 (10  $\mu$ g/mL) and variable concentrations of LL-37 (C), constant LL-37 (10  $\mu$ g/mL) and variable concentrations of LL-37 (C), constant LL-37 (10  $\mu$ g/mL) (E). (A-D) CXCL8 secretions were assessed by ELISA after 4 h. (E) *CXCL8* mRNA synthesis was quantified using qPCR over 4 h (top graph), and protein secretions were determined in cell supernatant for up to 16 h (bottom graph). (F) BMMs from *Camp*<sup>+/+</sup> and *Camp*<sup>-/-</sup> mice were challenged with LPS (1  $\mu$ g/mL) for 16 h. TNF- $\alpha$  and IL-6 secretions were then quantified using ELISAs. Data are means ± SEM (*n*= 3 independent experiments done in triplicate). *P*< 0.05 (One-way ANOVA *post hoc* Bonferroni correction) was considered significant. N.D= 'not detected'.

# 3.2. LPS and LL-37 through extracellular interaction induced CXCL8 secretion via

# TLR4.

Cathelicidin modulate colonic epithelial TLR4 expression in the presence of LPS derived

from either Salmonella spp. or E. coli<sup>163, 164</sup>. Herein, we detected a key role of colonic

epithelial TLR4 in cathelicidin-induced CXCL8 secretion; namely, inhibition of TLR4 with

LPS-RS blocked CXCL8 mRNA and protein synthesis in HT29 cells challenged with

LPS+LL-37 (Figs 3.6A-B). Further, TLR4 KD (ShTLR4) HT29 cells had diminished

CXCL8 production when stimulated with LL-37+LPS compared to sham-transfected controls

(**Fig 3.6C**). The TLR4 co-adaptor protein MD-2 was not required for production of CXCL8, as determined by the selective pharmacological MD-2 antagonist, L48H37 (**Fig 3.6D**). Secretion of the TLR4 accessory LBP was negligible in HT29 cells, irrespective of treatment (data below detection levels) and therefore could not account for the action of LPS/cathelicidin complex. We thus concluded that the combined action of LPS+cathelicidin was due to activation of TLR4.



Figure 3.6. TLR4 dependent CXCL8 synthesis in colonic epithelial cells stimulated with cathelicidin and LPS. (A-D) HT29 cells used were either normal (A-B and D-E) or sham transfected/knocked-down in TLR4 (C). (A-D) Cells were pre-treated with LPS-RS (5  $\mu$ g/mL) (or not; in C) or L48H37 (10  $\mu$ M) (D) followed by LPS (1  $\mu$ g/mL) and LL-37 (10  $\mu$ g/mL) alone or in combination. (E) Cells were treated by a mix of either LPS (1  $\mu$ g/mL) or LL-37 (10  $\mu$ g/mL) (1 h, 37°C) followed by substitution with polymyxin B (Poly B; 10  $\mu$ g/mL) or LPS (1  $\mu$ g/mL)+Poly B/bovine serum albumin (BSA;10  $\mu$ g/mL) (1 h, 37°C) followed by qPCR. *GAPDH* was used as negative controls. (A) *CXCL8* mRNA levels were assessed by qPCR. *GAPDH* was used as housekeeping control. (B-E) CXCL8 secretion was quantified by ELISA after 4 h. Data are means ± SEM (*n*= 3 independent experiments done in triplicate). *P* < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) was considered significant.

The next step was to determine if LPS binds to LL-37 to form a non-covalent complex prior to interacting with its colonic epithelium target. To this end, we first treated LPS with polymyxin B, a cyclic cationic polypeptide that binds to and neutralizes LPS. Prior treatment of LPS with polymyxin B prevented its ability to work in combination with cathelicidin to stimulate CXCL8 production by HT29 cells (**Fig 3.6E**). Conversely, if LPS and cathelicidin were first allowed to interact, followed by addition of polymyxin B, the combined action of LPS+cathelicidin to enhance CXCL8 production in HT29 cells was not affected (**Fig 3.6E**). These data pointed to extracellular formation of a polymyxin B resistant complex between LPS and LL37 to stimulate CXCL8 synthesis.

# **3.3. LL-37** promoted CXCL8 synthesis via monosialotetrahexosylganglioside (GM1)lipid raft mediated LPS uptake and intracellular TLR4 signalling.

We inferred that LL-37 interacted with LPS prior to its activation of TLR4 to induce CXCL8 production; therefore, we sought to determine how this complex reaches TLR4, which can have an intracellular location<sup>103, 165</sup>. We speculated that the LPS-cathelicidin complex might be internalized, as cathelicidin can act as a cell-penetrating peptide carrying cargo into cells<sup>166</sup>. Additionally, the cathelicidin sequence has a distant relationship to the cholera toxin B-subunit, known to internalize the toxin via ganglioside GM1 cell surface binding<sup>167, 168</sup>. We therefore hypothesized that LL-37+LPS complexes interact with lipid rafts and associated membrane GM1 ganglioside to internalize into epithelia and promote intracellular TLR4 signalling. For this, we first confirmed intracellular TLR4 expression in HT29 cells was higher (~3 fold) than cell surface expression (**Fig 3.7(A-B**)). Additionally, surface staining of TLR4 diminished in HT29 cells challenged with LPS+LL-37, but not with LPS or LL-37 alone (**Fig 3.7C**). Alexa488-conjugated-LPS in combination with LL-37 was time-

dependently internalized into HT29 cells (**Fig 3.7(D-E**)), but this did not occur with LPS alone or LPS in the presence of sLL-37 (**Fig 3.7D**).



Figure 3.7. Intracellular TLR4 expression and cathelicidin-mediated LPS uptake in HT29 cells. (A-B) TLR4 expression in non-permeabilized (surface) and permeabilized (intracellular) HT29 cells as assessed by fluorescence microscopy using anti-TLR4 antibody (1:100; yellow) and a histogram of flow cytometery based quantification using PE-labelled anti-TLR4-CD284 antibody (0.5 µg/mL). Isotype mouse IgG was used as a negative control in flow cytometery. (C) Fluorescent images depicting TLR4 localization in nonpermeabilized HT29 cells after LPS (1  $\mu$ g/mL)  $\pm$  LL-37 (10  $\mu$ g/mL) for 4 h (anti-TLR4 antibody; yellow). Bar graph representing quantification of TLR4 fluorescence as MFI. (D) HT29 cells were challenged with a combination of Alexa488-conjugated-LPS (1 µg/mL) and LL-37 (10 µg/mL), sLL-37 (10 µg/mL) or not for variable intervals (up to 4 h) followed by quantification of fluorescence. (E) Higher magnification picture of Alexa488-conjugated-LPS uptake in presence of cathelicidin at 4 h. WGA was used as a membrane marker. (C-D) TLR4 concentrations and LPS uptake were assessed using immunocytochemistry and quantified using ImageJ 1.50i software. Fluorescence was calculated and presented as MFI for three independent experiments. Data are means  $\pm$  SEM (n=3 independent experiments, done in triplicate). P < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparisons or two-tailed Student's *t*-test for two groups) were considered significant.

Addition of exogenous GM1 (100 µg/mL) to compete with LL-37+LPS for lipid rafts

reduced CXCL8 secretion (Fig 3.8A). Moreover, lipid raft disruption with a mixture of

methyl- $\beta$ -cyclodextrin (500  $\mu$ M) and mevinolin (250 ng/mL) (MM)<sup>86</sup> abolished the ability of

LPS+LL-37 to induce CXCL8 secretion (**Fig 3.8B**) and internalization of Alexa488conjugated-LPS (**Fig 3.8C**). Other endocytic pathways, including dynamin GTPasedependent or actin-dependent processes, assessed by pharmacological inhibition with dynamin inhibitor (D15) or actin assembly inhibitor cytochalasin D (CytoD) respectively, were not involved in CXCL8 synthesis (**Fig 3.8B**) or uptake of Alexa488-conjugated-LPS by HT29 epithelia (only CytoD; **Fig 3.8C**). Thus, LPS+LL-37 synergism in promoting CXCL8 production by colonic epithelial cells was dependent on extracellular binding with GM1 containing lipid rafts.



Figure 3.8. LL-37-mediated GM1-lipid raft dependent LPS uptake regulated CXCL8 synthesis in HT29 cells. (A) CXCL8 was quantified by ELISA in supernatants from HT29 cells treated with LPS and LL-37  $\pm$  exogenous GM1 (100 µg/mL). GM3 (100 µg/mL) was used as a negative control. (B-C) HT29 cells were pre-treated with a mix of lipid raft inhibitors (MM: methyl- $\beta$ -cyclodextrin (500 µM) and mevinolin (250 ng/mL)), or endocytosis inhibitors CytoD (2 µM), or dynamin-dependent endocytosis inhibitor (D15; 10 µM; only for (B)) for 1 h, followed by stimulation with unconjugated LPS (1 µg/mL) (B) or Alexa488-conjugated-LPS (1 µg/mL) (C) and LL-37 (10 µg/mL), either alone or in combination for 4 h. (B) CXCL8 protein secretion was quantified using ELISA. (C) LPS

uptake was assessed using immunocytochemistry and quantified using ImageJ 1.50i software. Fluorescence was calculated and presented as MFI for three independent experiments. Data are means  $\pm$  SEM (n= 3 independent experiments, done in triplicate). P < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparisons or two-tailed Student's *t*-test for two groups) were considered statistically significant.

# 3.4. MEK1/2 and miR-31-mediated NF-кВ activation drove colonic CXCL8

# transcriptional production induced by LL-37/LPS.

Activation of NF-κB is essential for CXCL8 synthesis in airway and cervical epithelial cells<sup>169</sup>. In agreement, we determined LPS+LL-37 induced time-dependent phosphorylation of the p65-NF-κB subunit in HT29 cells (**Fig 3.9A**). Similarly, in macrophages, LPS induced NF-κB (p65) activation was higher in *Camp*<sup>+/+</sup> BMMs compared with *Camp*<sup>-/-</sup> BMMs (**Fig 3.9B**). To validate the role of NF-κB in CXCL8 synthesis, we used a pharmacological inhibitor of the NF-κB activating IκB kinase β complex (PS1145). NF-κB inhibition blocked up-regulation of *CXCL8* mRNA and protein secretion in HT29 cells (**Fig 3.9(C-D)**). Further, we assessed ability of LL-37+LPS to stimulate NF-κB promoter elements upstream of CXCL8 in HT29 epithelial cells using a luciferase reporter construct<sup>170</sup>. Luciferase reporter activity was abolished upon mutation of the NF-κB promoter site, whereas it remained active after mutations of the AP-1 or NF-IL-6 sites (**Fig 3.9E**). This LPS+LL-37 mediated NF-κB p65 subunit phosphorylation was partially, albeit significantly, down-regulated in TLR4 knock-out cells (**Fig 3.9F**).



Figure 3.9. NF-KB activation in HT29 and BMMs and its requirement in CXCL8 mRNA synthesis induced by cathelicidin and LPS in HT29 cells. (A) HT29 cells or (B) BMMs from  $Camp^{+/+}$  and  $Camp^{-/-}$  mice were challenged with either (A) a combination of LPS (1 µg/mL) and LL-37 (10 µg/mL) for variable intervals or (B) LPS (1 µg/mL) alone for 2 h. (C-D) HT29 cells were pre-treated with the NF- $\kappa$ B inhibitor PS1145 (1  $\mu$ M) for 1 h, followed by treatment with LPS (1 µg/mL) and LL-37 (10 µg/mL). (C) CXCL8 gene synthesis was quantified after 2 h using qPCR (n=4) and (**D**) protein secretion was assessed after 4 h using ELISA. (E) HT29 cells were transfected with 174bp wild type (FL) or mutant CXCL8 promoter construct (containing individual mutated sites for either NF-κB or AP-1 or NF-IL-6) upstream of the luciferase gene, followed by stimulation with LPS (1 ug/mL) and LL-37 (10 µg/mL) for 4 h. Data are represented as fold change in RLU, normalized to renilla luciferase transfection control. IL-1 $\beta$  (10 ng/mL) was used as positive control. (F) HT29 cells were sham transfected/knocked down for TLR4 (ShTLR4) and treated with LPS+LL-37 treatment, either alone or in combination for 2 h. (A-B and F) p65 phosphorylation was assessed and quantified (not in **B**) by western blotting with specific antibodies. Total loading was confirmed after blotting for GAPDH. Data are means  $\pm$  SEM (n=3 independent experiments done in triplicate). P < 0.05 (one-way ANOVA post hoc Bonferroni correction for multiple group comparison or two-tailed Student's t-test for two groups) were considered statistically significant.

To delineate the molecular pathway upstream of NF- $\kappa$ B, we pharmacologically inhibited a variety of signalling proteins, including Src kinase, EGFR kinase, p38MAPK and MEK1/2 in HT29 cells. Inhibition of Src, EGFR kinase and p38MAPK had no effect on LL-37+LPS induced phosphorylation of NF- $\kappa$ B/p65 (**Fig 3.10A**). However, LL-37+LPS enhanced phosphorylation of MEK1/2 (**Fig 3.10B**) and that inhibition of MEK1/2 (by PD98059)

blocked phosphorylation-transactivation of the NF-κB subunit p65 (**Fig 3.10C**) in HT29 cells exposed to LL-37+LPS, with a concomitant reduction in CXCL8 secretion (**Fig 3.10D**).



**Figure 3.10.** MEK1/2 kinase-dependent NF-κB activation was required for CXCL8 synthesis induced by cathelicidin and LPS in colonic epithelial cells. (A-D) HT29 cells were treated with inhibitors for EGFR kinase, Src kinases, p38MAPK (A) and MEK1/2 (C and D) (AG1478 (1 μM), PP1 (1 μM), SB203580 (2 μM), and PD98059 (20 μM), respectively) or not (B) for 1 h, followed by LPS+LL-37 treatment, alone or in combination for 2 h (A-C) or 4 h (D). (A-C) Western blotting was performed to assess and quantify p65 (A and C) or ERK1/2 phosphorylation (B) with specific antibodies. (D) CXCL8 secretion was quantified by ELISA. Total loading was confirmed after blotting for GAPDH. (Data are means ± SEM (*n*= 3 independent experiments done in triplicate). *P* < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) were considered significant.

Thus, cathelicidin and LPS increased CXCL8 mRNA synthesis via NF-KB/p65 activation,

which is downstream of TLR4 and MEK1/2 signalling molecules.

# 3.5. LL-37/LPS complex initiates TLR4/Src-EGFR-p38MAPK axis to promote CXCL8 stability.

Intracellular signalling required for CXCL8 synthesis in colonic epithelium involves protein tyrosine kinases and MAPKs<sup>171</sup>, but the detailed interactive pathway is elusive. In our study, LPS+LL-37 treatment resulted in a time-dependent increase in phosphorylation of the EGFR and p38MAPK (increasing up to 2 and 4 h for EGFR and p38MAPK, respectively) (Fig **3.11(A-B)**), which was stronger than the effect promoted by either stimuli alone (data shown for 2 h; Fig 3.11C). Next, we focussed on how TLR4 and EGFR may crosstalk to facilitate p38MAPK phosphorylation in the LPS+LL-37 induced CXCL8 secretion. Of interest was the Src family LYN kinase, as it is linked to TLR4 and EGFR in human mammary epithelial cells in LPS-induced (ip) lethality<sup>172</sup>. Inhibition of EGFR (by AG1478) as well as of Src (by PP1) reduced activation of EGFR and p38MAPK (Fig 3.11C) and CXCL8 secretion in HT29 epithelia stimulated by LPS+LL-37 (Fig 3.11D). Intriguingly, inhibition of EGFR did not affect CXCL8 gene synthesis in LPS+LL-37 treated HT29 cells (Fig 3.11E). We inferred LPS+LL-37 could promote CXCL8 secretion by regulating CXCL8 mRNA half-life and not directly by transcript synthesis. To test this, we used an actinomycin D chase assay and determined that CXCL8 mRNA was stable for 1 h when HT29 cells were treated with actinomycin D alone, declined at 2 h (~40% of control level) and stabilized (~25%) over 3-4 h (Fig 3.11F). In contrast, inhibition of p38MAPK accelerated the decline in CXCL8 transcripts within 30 min (~60% of control level) and 1 h (~40%) (Fig 3.11F).



Figure 3.11. Src-EGFR kinase mediated p38MAPK signalling promoted CXCL8 mRNA stabilization in colonic epithelial cells stimulated by cathelicidin and LPS. (A-B) LPS+LL-37 mediated time-dependent activation of EGFR (A) and p38MAPK (B) in HT29 epithelia as detected by Western blotting with specific antibodies. Total loading was confirmed after immunoblotting with EGFR and p38MAPK, respectively. (C-E) HT29 cells were pre-treated with AG1478 (1 µM) or the Src kinases inhibitor PP1 (1 µM; only for (C-**D**)) for 1 h, followed by treatment with LPS (1  $\mu$ g/mL) and LL-37 (10  $\mu$ g/mL). (C) Activation of p38MAPK was assessed after 2 h using Western blotting for phosphorylated p38MAPK. CXCL8 (**D**) protein secretion (n=4) was assessed after 4 h using ELISA and (**E**) mRNA synthesis was quantified after 2 h using qPCR (n=4). (F) HT29 cells were stimulated with LPS (1  $\mu$ g/mL) and LL-37 (10  $\mu$ g/mL) for 2 h, washed, and incubated with a gene synthesis inhibitor actinomycin D (ActD 1  $\mu$ M) ± a p38MAPK inhibitor SB203580 (2  $\mu$ M) for different time periods (n=4). Total RNA was extracted and quantified using qPCR. Data are means  $\pm$  SEM (n=3 independent experiments done in triplicate, unless mentioned otherwise in respective sub-figure). P < 0.05 (two-tailed Student's *t*-test for two groups) was considered significant.

```
Furthermore, to determine if other known membrane based receptors for LL-37 were
involved in synergistic response with LPS, we pre-treated HT29 cells with a variety of
chemical inhibitors. Pharmacological inhibition of LL-37 receptors, including FPRL-1,
P2X7, MMP and EGFR 2/ErbB2<sup>173</sup> did not affect synergistic action of cathelicidin and LPS
to stimulate CXCL8 secretion (Fig 3.12). Taken together, we inferred that synthesis of
CXCL8 protein in colonic epithelium via synergism between LPS and cathelicidin was
```

contingent on two pathways: NF- $\kappa$ B downstream of TLR4 and MEK1/2 MAPK for *CXCL8* mRNA synthesis, plus another NF- $\kappa$ B-independent event, regulated by Src, the EGFR kinase, and p38MAPK that stabilizes *CXCL8* mRNA.



Figure 3.12. Cathelicidin and LPS dependent CXCL8 secretion in colonic epithelial cells in presence of various inhibitors. HT29 cells were pre-treated with inhibitors TAK165 (ErbB2; 10  $\mu$ M), WRW4 (FPRL1; 10  $\mu$ M), GM6001 (MMP; 10  $\mu$ M) and A740003 (P2X7; 10  $\mu$ M) followed by treatment with LPS and LL-37, either alone or in combination. CXCL8 protein secretion was determined using ELISA after 4 h (n= 4). Data are means ± SEM (*n*= 3 independent experiments done in triplicate, unless mentioned specifically in respective figure). *P* < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) was considered significant.

### 3.6. Cathelicidin was essential for neutrophil recruitment, activation and pathogen

### clearance in infectious colitis.

Migration of neutrophils into inflamed tissue is a critical part of the local innate immune protection. Human CXCL8 and murine CXCL1 are effective chemokines acting via CXCR1/2 receptors expressed on neutrophils<sup>174</sup>. Since there was increased CXCL1 secretion in colonic mucosa of *Camp*<sup>+/+</sup> than *Camp*<sup>-/-</sup> mice upon *C. rodentium* infection, we wanted to determine corresponding neutrophil infiltration. *Camp*<sup>-/-</sup> mice during *C. rodentium* infection lacked neutrophil infiltration into the distal colon compared to *Camp*<sup>+/+</sup> mice (7 d pi), as determined by immunofluorescence staining (**Fig 3.13A**) and MPO activity (**Fig 3.13B**).

Whereas the epithelial damage marker lipocalin-2 protein expression was similarly increased during *C. rodentium* infection (**Fig 3.13C**), *Camp<sup>-/-</sup>* mice had reduced activation (phosphorylation) of EGFR and NF- $\kappa$ B (p65) in their colons (**Fig 3.13D**). Fecal shedding of *C. rodentium* was significantly greater (~8-fold) in *C. rodentium* infected *Camp<sup>-/-</sup>* mice compared to infected *Camp*<sup>+/+</sup> mice at 3, 5 and 7 d pi (**Fig 3.13E**). *C. rodentium* was isolated from the spleens and livers of all *Camp<sup>-/-</sup>* mice but only in 50% of *Camp*<sup>+/+</sup> mice (both for spleen and liver) (**Fig 3.13F**).



Figure 3.13. Neutrophil activation, fecal shedding and extra-intestinal bacteria in *Camp*<sup>+/+</sup> and *Camp*<sup>-/-</sup> mice infected by *C. rodentium*. (A-D) C57BL/6 *Camp*<sup>+/+</sup> and *Camp*<sup>-/-</sup> mice were orally infected with C. rodentium (1 x  $10^8$  CFU in 200 µL of PBS) for 7 days. (A) Immunofluorescence staining of mouse colonic tissue with anti-Lv6G antibody (5 µg/mL). Neutrophils were widely distributed in colon (lumen, crypt and lamina propria) however, this representative image only shows neutrophils along the crypts (yellow arrows) and in lamina propria (white arrows). Fluorescence was calculated using ImageJ 1.50i software and represented as MFI. (B-C) Bar graphs depicting MPO activity (B) and lipocalin-2 secretions in feces (C) in murine colonic mucosa after 7 dpi with C. rodentium. Data are absolute values (MPO activity (U/g) and ng/mL for lipocalin-2) with respect to  $Camp^{+/+}$  PBS control. (D) Western blotting for phosphorylated EGFR and p65 subunit of NF-kB in tissue lysates from distal colon of Camp<sup>+/+</sup> and Camp<sup>-/-</sup> mice. (E-F) C57BL/6 Camp<sup>+/+</sup> and Camp<sup>-/-</sup> mice were infected by C. rodentium (1 x 10<sup>8</sup> CFU in 200 µL of PBS), followed by collection of fecal pellets at day 3, 5 and 7 pi (E) and liver and spleen at day 7 (F) post-infection (n=4mice/group). Fresh fecal pellets (1/per mouse), homogenized liver or spleen were serially diluted in sterile PBS, plated on McConkey agar and counted to obtain CFU/g. (E) Data were

then normalized to infected C57BL/6  $Camp^{+/+}$  mice and represented as fold change in CFU/g of feces. One fold change represents ~ 10<sup>5</sup>, 10<sup>6</sup> and 10<sup>7</sup> CFU/g for day 3, 5 and 7 pi, respectively. Data are means ± SEM (n= 4 mice/group). P < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) was considered significant.

Using naïve human neutrophils, we determined that secreted products released by the HT29 cells in response to LPS+LL-37 promoted secretion of neutrophil elastase (**Fig 3.14A**) and increased calcium flux (**Fig 3.14B**). This response was mostly attributed to CXCL8, as anti-CXCL8 antibody and a CXCL8 receptor antagonist blocked both events (**Figs 3.14A and 3.14B**). Taken together, endogenous CRAMP, by inducing the chemoattractant CXCL1, contributed to the influx of neutrophils into the colon and *C. rodentium* clearance.



Figure 3.14. Secreted CXCL8 from colonic epithelium stimulated by cathelicidin and LPS induced calcium flux and activation of human primary neutrophils. (A-B) Human neutrophils were seeded at (A) 1 x 10<sup>4</sup>/well in an eight-well chamber and incubated at 37° for 1 h or (B) 1 x 10<sup>6</sup> cells and pre-incubated with Fluo4-NW dye (45 m, RT). (A-B) Cells were then exposed to supernatant from HT29 epithelia unstimulated (control) or stimulated with LPS (1 µg/mL) and LL-37 (10 µg/mL), either alone or in combination, for 4 h ± anti-CXCL8 antibody (1 µg/mL) or CXCR1/2 inhibitor SCH527123 (20 µM). rCXCL8 was used as positive control. IgG was used as an isotype control. (A) Neutrophil activation was assessed by neutrophil elastase secretion (depicted by white arrows) using immunocytochemistry using anti-neutrophil elastase antibody (5 µg/mL). Data are fold increase in MFI normalized to respective control, for three independent experiments. (B) Data are percentage fluorescence emission at 530 nm of positive control calcium ionophore A23187 (CI A23187). Data are means ± SEM (*n*= 3 independent experiments done in triplicate). *P* < 0.05 (one-way ANOVA *post hoc* Bonferroni correction for multiple group comparison or two-tailed Student's *t*-test for two groups) was considered significant.

Study 2. Cathelicidin induced Tollip synthesis and prevented TLR-dependent apoptosis in colonic epithelium.

# 3.7. Cathelicidin prevented apoptosis in colonic epithelium via Tollip.

Cathelicidin prevents TLR4-dependent apoptosis in endothelial cells, spontaneous apoptosis in neutrophils, and apoptosis in cardiomyocytes exposed to ischemia/reperfusion mediated injury<sup>64, 175, 176</sup>. Tollip, a negative regulator of TLR/IRAK signalling, inhibited apoptosis in intestinal epithelial (INT407) cells exposed to TNF- $\alpha$  and IFN- $\gamma^{60}$ . Whether cathelicidin exerts anti-apoptotic effects in colonic epithelium via Tollip remained unknown. Using normal HT29 cells and HT29 cells depleted of Tollip (ShTollip; **Fig 3.15A**), we reported that the combination of IFN- $\gamma$  (300 U/mL) and TNF- $\alpha$  (variable doses) induce colonic apoptosis (**Figs 3.15B and 3.15C**), as determined by assessment of cleaved caspase-3 protein band on a Western blot (**Fig 3.15B**) and apoptotic secretions as measured by death ELISA in normal HT29 cells and ShTollip (**Fig 3.15C**). Further, pre-treatment with LL-37 (8 h) reduced IFN- $\gamma$ and TNF- $\alpha$  induced caspase-3 and PARP cleavage (**Fig 3.15D**) and cell death in normal HT29 cells as determined by LDH assays (**Fig 3.15E**). Such prevention of apoptosis by cathelicidin was not observed in ShTollip cells (**Fig 3.15D and 3.15E**).



**Figure 3.15. Cathelicidin prevented apoptosis in HT29 cells.** (A-E) HT29 cells were either sham-transfected or knocked-down in Tollip (ShTollip). (A) Western blot depicting significant knock-down of Tollip protein in ShTollip cells. (B-E) Colonic HT29 epithelial cells were either not pre-treated (B-E) or pre-treated with cathelicidin LL-37 (10 µg/mL; 8 h) (D-E) followed by treatment either with variable concentrations of TNF- $\alpha$  and IFN- $\gamma$  (300 U/mL) (B-C) or TNF- $\alpha$  (100 ng/mL) and IFN- $\gamma$  (300 U/mL) (D-E) for 18 h. (B and D) Western blot depicting levels of cleaved caspase-3 and cleaved PARP (only in B). (C) Apoptosis was quantified using ELISA. (E) A bar graph showing LDH activity as marker of cell death in cell supernatant. Data are fold change in LDH activity normalized to control untreated group. (A-B and D) GAPDH was used as housekeeping control. (C) Data were represented as Enrichment factor i.e. ratio of treatment <sub>(OD)</sub> and control <sub>(OD)</sub>. Data are means ± SEM (*n*= 3 independent experiments done in triplicate, unless mentioned otherwise in respective sub-figure). *P* < 0.05 (two-tailed Student's *t*-test for two groups) was considered significant.

To further assess the role of endogenous cathelicidin in prevention of colonic apoptosis,

 $Camp^{+/+}$  and  $Camp^{-/-}$  mice were infected with *C. rodentium* (5 x10<sup>8</sup> CFU/mouse). Distal colon from infected  $Camp^{+/+}$  mice had lower TUNEL staining, mostly restricted to epithelial cells, when compared to  $Camp^{-/-}$  mice at 7 d pi (**Fig 3.16A**). Likewise, CECs isolated from *C. rodentium* infected  $Camp^{+/+}$  mice had lower caspase-3 and PARP cleavage compared to cells from corresponding  $Camp^{-/-}$  mice (**Fig 3.16B**). Therefore, we inferred that cathelicidin

prevented apoptosis in colonic epithelium during *C. rodentium* infection, at least in part, by increasing Tollip protein.



Figure 3.16. Cathelicidin prevented apoptosis in colonic epithelium of  $Camp^{+/+}$  and  $Camp^{-/-}$  mice challenged with *C. rodentium*. (A-B)  $Camp^{+/+}$  and  $Camp^{-/-}$  mice were challenged with *C. rodentium* (5 x 10<sup>8</sup> CFU in 200uL PBS) for 7 d (n= 4/group). (A) Distal colonic tissues were assessed for apoptosis using TUNEL staining (results communicated with a bar). (B) CECs isolated from infected mice were blotted for cleaved caspased-3 and cleaved PARP. GAPDH was used as housekeeping control for Western blotting. Data are shown as means  $\pm$  SEM (*n*= 4 mice/group, unless mentioned otherwise in respective sub-figure). *P* < 0.05 (two-tailed Student's *t*-test for two groups) was considered significant.

# 3.8. Cathelicidin induced Tollip synthesis in hematopoietic and non-hematopoietic cells

# in the colon.

Cathelicidin modulates colonic mucosal sensing by driving TLR responses in leukocytes and epithelial cells in the colon. LL-37 inhibited TLR-2 and -4 dependent NF-κB activation in *E. coli* infected murine (J774) macrophages<sup>177</sup>, whereas, in the presence of synthetic ODN, LL-37 diminished TLR-9 production in HT29 cells<sup>109</sup>. Since mechanisms used by this peptide to influence TLR signalling are poorly described, we investigated whether cathelicidin

negatively regulated TLRs via Tollip. Synthetic LL-37 did not increase Tollip mRNA expression in HT29 cells (Fig 3.17A) but did induce Tollip protein expression  $(0.2 - 2 \mu M)$ (Fig 3.17B). Of note, effect of cathelicidin on Tollip only occurred at low doses ( $< 2 \mu M$ ), whereas higher concentrations of LL-37 (>2  $\mu$ M) had either no effect or reduced the amount of Tollip (e.g., as noticed at ~40  $\mu$ M) (Fig 3.17C). Likewise, whereas both Camp<sup>+/+</sup> and Camp<sup>-/-</sup> CECs showed no increase in Tollip mRNA (Fig 3.17D), enhanced constitutive Tollip protein expression (Fig 3.17E) occurred in  $Camp^{+/+}$  compared to  $Camp^{-/-}$  CECs. This increased Tollip protein expression in colons of  $Camp^{+/+}$  mice was detected mostly in epithelial cells at the top of the crypts but not in other types of cells (Fig 3.17F). A similar effect of cathelicidin inducing Tollip was also demonstrated in murine colonoids derived from  $Camp^{+/+}$  mice (Fig 3.17G). Among other cells, we tested BMMs and murine (L929) fibroblasts cells for Tollip protein synthesis. *Camp*<sup>+/+</sup> BMMs expressed higher Tollip protein as compared to Camp<sup>-/-</sup> controls (Fig 3.17G and 3.17H, respectively). Likewise, L929 fibroblasts also had increased Tollip expression after stimulation with LL-37 whereas LPS did not affect Tollip expression (Fig 3.17I). Thus, cathelicidin promoted Tollip expression in both hematopoietic and non-hematopoietic cells in the colon, including epithelia, fibroblasts and leukocytes.



**Figure 3.17. Cathelicidin promoted Tollip protein synthesis.** (A-C) HT29 cells were treated with cathelicidin LL-37 at 10 µg/mL (2 µM) for variable intervals (A-B) or varied concentrations for 8 h (C). (A) A bar graph depicting *Tollip* gene expression as assessed by qPCR. (B-C) Western blots showing Tollip protein synthesis. (D-F) Tollip expression was assessed in *Camp*<sup>+/+</sup> and *Camp*<sup>-/-</sup> mice, both in isolated CECs (D-E) and in distal colonic tissue (F) (n= 4/group). (D) *Tollip* gene expression as assessed by qPCR. Western blot (E) and immunohistochemistry (F) showing Tollip protein expression in mice. (G-I) Tollip protein synthesis was assessed using Western blotting in murine colonoids (G), murine BMMs (H) and L929 murine fibroblasts (I), either upon LL-37 (10 µg/mL; variable time) treatment (G and I) or as a function of endogenous cathelicidin expression (H). GAPDH was used as normaliser both for qPCR and Western blotting. Data are means ± SEM (*n*= 3 independent experiments done in triplicate, unless mentioned otherwise in respective sub-figure). *P* < 0.05 (two-tailed Student's *t*-test for two groups) was considered significant.

## 3.9. Cathelicidin prevented IRAK-1 phosphorylation and autocrine expression of pro-

### inflammatory cytokines in colonic epithelium via Tollip.

IRAK-1 is a natural ligand of Tollip; dissociation of Tollip allows IRAK-1 phosphorylation and induction of pro-inflammatory genes in response to IL-1 $\beta^{115}$  or LPS stimulation<sup>178</sup> in human embryonic kidney (293T) cells. Thus, we wanted to determine whether cathelicidininduced Tollip would prevent IRAK-1 phosphorylation by LPS. Pre-treatment with LL-37 prevented phosphorylation of IRAK-1 after LPS exposure in HT29 cells (**Fig 3.18A**). Further, normal HT29 cells had lower basal IRAK-1 phosphorylation compared to HT29 cells silenced in LL-37 (ShLL-37) and Tollip (ShTollip) genes (**Fig 3.18B**). These findings were corroborated in CECs isolated from  $Camp^{+/+}$  and  $Camp^{-/-}$  mice wherein, there was a constitutively lower IRAK-1 phosphorylation in CECs of  $Camp^{+/+}$  mice compared to those in  $Camp^{-/-}$  mice (**Fig 3.18C**). As Tollip negatively regulates cytokine expression, we determined whether absence of Tollip would enhance expression of pro-inflammatory genes in LL-37 treated HT29 cells. Gene expression of *IFN-γ*, *TNF-α* and *IL-1β* was increased in ShTollip cells stimulated with LL-37, but not in normal HT29 cells expressing Tollip (**Fig 3.18(D-F**)). Taken together, negative feedback from Tollip protein and subsequent inhibition of IRAK-1 phosphorylation seemed to prevent cathelicidin-induced pro-inflammatory responses in colonic epithelium.



**Figure 3.18.** Cathelicidin prevented IRAK-1 phosphorylation and pro-inflammatory cytokine expression in colonic epithelium. (A-C) Phosphorylated IRAK-1 levels were assessed using Western blotting in colonic HT29 epithelial cells (A-B) and isolated CECs from  $Camp^{+/+}$  and  $Camp^{-/-}$  mice (n= 5/group) (C). HT29 cells were either (A) pre-treated with cathelicidin LL-37 (10 µg/mL; 8 h) followed by treatment with LPS (variable concentration; 1 h) or (B) knocked down in *LL-37* or *Tollip*. (D-F) Relative gene expressions for IFN-γ (D), TNF-α (E) and IL-1β (F) were assessed in sham transfected and *Tollip* KD cells upon treatment with LL-37 (10 µg/mL; 8 h) followed by washing and then, left

untreated in serum free media for 16 h. Data are means  $\pm$  SEM (*n*= 3 independent experiments done in triplicate, unless mentioned otherwise in respective sub-figure). *P* < 0.05 (two-tailed Student's *t*-test for two groups) was considered significant.

# 3.10. Cathelicidin promoted Tollip synthesis via EGFR-Argonaute 2 (AGO2) mediated

# inhibition of mir-31 maturation

LL-37 signals through multiple cellular receptors including EGFR, FPR2 and P2X7, on cells such as intestinal epithelium, keratinocytes and leukocytes<sup>179</sup>. We wanted to determine which of these three receptor(s) was involved in cathelicidin induced Tollip synthesis. Cathelicidin time-dependently phosphorylated EGFR in HT29 cells (**Fig 3.19A**). This induced EGFR activation (phosphorylation) was required for LL-37-mediated Tollip production, as demonstrated by pharmacologically blocking EGFR (AG1478). Moreover these effects were selective for LL-37, as they were not observed with the sLL-37 (**Fig 3.19B**). Neither FPR2 nor P2X7 appeared to have a role in cathelicidin-mediated Tollip synthesis (**Fig 3.19C**).



Figure 3.19. Cathelicidin promoted EGFR-AGO2 mediated mir-31 maturation in HT29 cells. (A-C) HT29 cells were pre-treated with inhibitors for EGFR kinase  $(1 \ \mu M)$  (B), P2X7  $(10 \ \mu M)$  and FPR2  $(10 \ \mu M)$  (C) (AG1478, A740003 and WRW4, respectively) or not (A),

followed by treatment with cathelicidin LL-37 (2  $\mu$ M) for variable time (A) or 8 h (B-C). Western blotting was performed to assess EGFR phosphorylation activation (A) and Tollip protein synthesis (B-C). GAPDH was used as normaliser. (A) EGF (1 ng/mL) was used as positive control. (D-E) Sham-transfected or mir-31 plasmid incorporated HT29 cells were assessed for miR-31 overexpression using qPCR (D), and Tollip protein synthesis using Western blotting (E). (F) HT29 cells treated with LL-37 (2  $\mu$ M) for variable time were assessed for relative quantification of miR-31 using qPCR. (G-H) miR-31 reporter plasmid was transfected into HT29 cells, followed by pre-treatment with EGFR kinase inhibitor (AG1478; 1 µM) (H) or not (G). Cells were then treated with LL-37 (2 µM) for variable time. Luciferase activity was assessed as a measurement of miR-31 expression levels. Data are represented as % miR-31 expression normalized to untreated control. (I) HT29 cells were pre-treated or not with EGFR kinase inhibitor (AG1478; 1 µM), followed by treatment with LL-37 (2 µM) for variable time. Western blot showing levels of phosphorylated AGO2 protein. GAPDH was used as housekeeping control. (D-F) U6 small nuclear RNA was used as control for miR-31 qPCR estimation. Data are means  $\pm$  SEM (n=3 independent experiments done in triplicate, unless mentioned otherwise in respective sub-figure). P < 0.05(two-tailed Student's *t*-test for two groups) was considered significant.

There was increased Tollip protein production but not Tollip gene synthesis upon LL-37 treatment in colonic epithelium. In this regard, post-translational regulation is key in Tollip synthesis in both hematopoietic and non-hematopoietic cells<sup>180, 181</sup>. LPS diminished the rate of Tollip protein turnover, as assessed by pulse chase experiment in pCMV-Tollip plasmid transfected THP-1 cells<sup>181</sup>. Mechanistically, miR-31 is of particular interest; it binds the nucleotide region between +1876 and + 2398 on the 3'-untranslated region of the Tollip gene and promotes translational repression of Tollip in intestinal epithelium<sup>180</sup>. As shown in Study I, LL-37 reduced levels of miR-31 in HT29 cells. Thus, we speculated that the role of cathelicidin in Tollip expression may involve post-transcriptional regulation by miRNA. Overexpression of mir-31 in HT29 cells (Fig 3.19D) prevented the ability of LL-37 to promote Tollip (for up to 8 h) (Fig 3.19E). Indeed, LL-37 reduced miR-31 expression in HT29 cells as assessed by qPCR (Fig 3.19F) and miR-31 luciferase-plasmid assay (Fig **3.19G**). Since we noticed LL-37 enhanced signalling through EGFR, we wanted to evaluate whether EGFR kinase was crucial in regulating miR-31 levels. Inhibition of EGFR (AG1478 inhibitor) in HT29 cells abolished the LL-37 mediated miR-31 expression down-regulation (Fig 3.19H). Mechanistically, LL-37 promoted phosphorylation-inactivation of AGO2, a

dicer protein involved in maturation of miRNAs (**Fig 3.19I**). Such AGO2 phosphorylation was shown by LL-37 to be EGFR dependent (**Fig 3.19I**). Likewise, *Camp*<sup>+/+</sup> CECs had constitutive higher phosphorylation of EGFR with a concomitant inactivation of AGO2 (phosphorylation) (**Fig 3.20A**) and reduced miR-31 expression in comparison to *Camp*<sup>-/-</sup> CECs (**Fig 3.20B**). Overall, cathelicidin mediated colonic Tollip expression via an EGFR-AGO2 signalling axis and by degrading miR-31.



Figure 3.20. Cathelicidin promoted EGFR-AGO2 activation and mir-31 maturation in in *Camp*<sup>+/+</sup> mice. (A-B) CECs isolated from  $Camp^{+/+}$  and  $Camp^{-/-}$  mice were assessed either for phosphorylated EGFR and AGO2 protein using Western blotting (A) or miR-31 expression levels using qPCR (B) (n= 5/group). GAPDH and U6 small RNA were used as housekeeping control for Western blotting and qPCR, respectively. Data are means ± SEM (*n*= 3 independent experiments done in triplicate, unless mentioned otherwise in respective sub-figure). *P* < 0.05 (two-tailed Student's *t*-test for two groups) was considered significant.

#### **CHAPTER 4- DISCUSSION**

# 4.1. Cathelicidin triggered neutrophil chemotactic factor CXCL8 synthesis in colonic epithelium in response to Gram negative LPS and promoted early recruitment of neutrophils.

This thesis provides novel information that cathelicidin/LL-37 has a physiological role in gut innate defenses by synergizing with bacteria-derived LPS to promote cellular production of the neutrophil chemoattractant, CXCL8 (murine CXCL1). This immunomodulatory function of cathelicidin is in addition to their role as a potential endogenous antimicrobial peptide that promotes bacterial elimination. The ability of LPS and cathelicidin working in concert (LPS+LL-37), but not individually, to activate TLR4 results in CXCL8-stimulated recruitment of disease-mitigating neutrophils to the site of invasion. The LPS+LL-37 driven epithelial chemokine response occurred at concentrations of LL-37 which are neither microbicidal<sup>182</sup> nor cytotoxic. The unusual mechanism whereby the extracellular LPS+LL-37 complex is internalized to reach its TLR4 intracellular target involves a ganglioside GM1mediated lipid raft process, akin to the way cholera toxin is internalized to act. The ability of cathelicidin to promote internalization of LPS was consistent with a report that cathelicidin can form aggregates with DNA, presumably via many positive charges in the cathelicidin sequence, to promote internalization of DNA into endosomal compartments wherein it triggers TLR9 signalling<sup>90</sup>.

In *Camp*<sup>-/-</sup> mice, endogenous cathelicidin are needed for controlling *C. rodentium* infection<sup>183,</sup> <sup>184</sup>. However, the implicit understanding to date has been that this action is due to antimicrobial actions of cathelicidin. We confirmed that elimination of *C. rodentium* was unequivocally impaired in the absence of cathelicidin. Furthermore, we provided a new paradigm for this action (**Fig 4.1**). Instead of causing microbial killing directly, we inferred

80

lipid raft-internalized cathelicidin/LPS-triggered activation of intracellular TLR4 to elevate production of the neutrophil chemokine CXCL8 (or murine CXCL1). Responding to this chemokine, we suggested that invading neutrophils become responsible for triggering the elimination of *C. rodentium*. Indeed, *Camp<sup>-/-</sup>* mice had a reduced capacity to produce specific chemokines (CXCL1 and CCL3), whereas others (CXCL9 and CXCL10) were increased in *Camp<sup>-/-</sup>* mice, likely as a compensatory event. Remarkably, CXCL1 chemokine secretion by colonic epithelium was necessary for neutrophil recruitment and activation in the gut. A timely CXCL1 dependent neutrophil recruitment in infected *Camp<sup>+/+</sup>* mice, whereas contributing to the inflammatory damage (crypt hyperplasia, colon length shortening), would restrict intestinal infection. Thus, defective gut defense in cathelicidin-null mice was, at least in part, attributed to a reduced CXCL1-stimulated influx of neutrophils in the setting of infectious colitis.

The synergistic effect of *S*. Typhimurium (live or heat-killed)/associated LPS+cathelicidin was confirmed in cultured intestinal epithelial cells with various phenotypes and functional characteristics (HT29, T84), as well as in wild type and *Camp*<sup>-/-</sup> mice, and in *ex vivo* murine colonoids. Of note, increased chemokine production did not occur with cathelicidin or LPS acting independently, but only when both agonists were combined. For instance, synthesis of CXCL8 in colonic epithelium (either mRNA transcription or protein translation) via phosphorylation of NF- $\kappa$ B (p65) did not occur with addition of LPS alone (for up to 4 h). Consistent with this, only high concentrations of LPS (up to 1 µg/mL for 18 h) increased CXCL8 production in previous studies with HT29 cells<sup>185</sup>. Further, ip administration of LPS did not affect colonic CXCL1 synthesis whereas LL-37 alone reduced CXCL1 levels in *Camp*<sup>-/-</sup> mice. It is likely that LPS does not increase CXCL1 in *Camp*<sup>-/-</sup> mice due to the absence of cathelicidin that is required to act together with LPS. In the case of LL-37 acting alone, perhaps lower concentrations of the peptide ( $< 20 \,\mu g/mL$ ) may induce expression of toll interacting-protein (Tollip, a negative regulator of TLRs), thereby reducing basal CXCL1 synthesis. This hypothesis was tested and confirmed in Study 2. Therefore, cathelicidin represented a multi-checkpoint strategy in the gut to prevent unwarranted inflammation in the presence of commensals and to activate the colonic epithelium (inducing CXCL8 synthesis) only during a challenge by Gram negative pathogen when LPS is readily available and expression of cathelicidin is enhanced to form a LPS+LL-37 complex. Likewise, Camp<sup>+/+</sup> BMMs had increased pro-inflammatory cytokine (TNF-α and IL-6) synthesis compared to  $Camp^{-/-}$  BMMs upon LPS challenge. By contrast, TNF- $\alpha$  secretion was reduced following LPS+LL-37 treatment of PMA-differentiated human THP-1 monocytes, although CXCL8 synthesis remained unaffected. Such diverse signalling between endogenous and exogenous cathelicidin was reported in macrophages, wherein exogenous murine cathelicidin CRAMP diminished LPS induced NF-kB activation but endogenous CRAMP promoted NF-kB activation<sup>186</sup>. Differential responses among cell types treated with LPS+LL-37 are possible due to the pleiotropic nature of cathelicidin acting in concert with multiple cell surface (EGFR, P2X7 and FPR2) and cytoplasmic (TLR9 and GAPDH) receptors<sup>187</sup>.

Mechanistically, our data pointed to a physical extracellular interaction between LL-37 and LPS, followed by internalization of an LPS+LL-37 complex via GM1-containing lipid rafts. Specific sites of interactions between LPS and LL-37, and for the interaction between cathelicidin and self-DNA<sup>90</sup>, remain to be determined. The interaction between LL-37 and other negatively charged ligands like LPS and DNA may involve multiple lysine (K)/arginine (R) residues in LL-37. Positive charges on cathelicidin in proximity to negatively charged LPS<sup>188</sup> would enable embedding of LL-37 hydrophobic helices into LPS micelles<sup>189</sup>. That said, it can be noted that of the 10 positive lysine/arginine side chains in LL-37, three are

situated immediately upstream of a negatively charged aspartic or glutamic acid, whereas four are present as dibasic clusters (FFR<sup>140</sup>K<sup>141</sup>S; FK<sup>151</sup>R<sup>152</sup>IVQRI). The two C-terminal LL-37 basic residues are spaced by four amino acids (LR<sup>162</sup>NLVPR<sup>167</sup>TES), one of which is a proline that would generate a 'twist' between the two arginines, thereby limiting a ligand interaction. Thus, if positive side-chains in LL-37 are involved in an LPS or DNA interaction, the most likely sequences would be FFR<sup>140</sup>K<sup>141</sup>S and FK<sup>151</sup>R<sup>152</sup>IVQRI. Both of these sequences, but not the C-terminal arginines of LL-37 (LR<sup>162</sup>NLVPR<sup>167</sup>TES), would also be present in cathelicidin-derived inflammatory peptide, FA-29<sup>190</sup>. Thus, both LL-37 and FA-29 could synergize with LPS to drive CXCL8/cytokine synthesis via TLR4. Future studies could test these hypotheses to identify the precise site whereby LPS interacts with LL-37 and to determine if FA-29, like cathelicidin, can synergize with LPS to drive cytokine production. Based on our data, the LPS+LL-37 complex gained access to intracellular TLR4 via a GM1/lipid raft mechanism. In support of this finding, lipid rafts were necessary for LPS signalling via intracellular TLR4 in murine small intestinal crypt (m-IC<sub>cl2</sub>) cells<sup>191</sup> and, lipid rafts were involved in the ability of LL-37 to promote endocytosis of LPS in lung epithelial cells<sup>86</sup>. Further work identifying the nature of the LL-37/LPS interaction should clarify the way in which the complex interacts with lipid raft-containing ganglioside GM1.

Both LPS trafficking and intracellular availability of LPS+LL-37 were crucial to enhance TLR4 signalling for CXCL8 secretion. Although polymyxin B enhanced LPS uptake by gut epithelia, it did not promote CXCL8 secretion, possibly because of the anti-endotoxin activity of polymyxin B. TLR4 occurs both on the surface and intracellular compartments in colonic epithelium<sup>103</sup>; however, based on our data, intracellular TLR4 was necessary for LPS+LL-37 to drive cytokine synthesis. Indeed, it has been hypothesized that intracellular TLR4 in the intestinal epithelium is strategically needed to avoid unwarranted inflammation<sup>192</sup>. Co-factors of TLR4, e.g. MD-2 and LBP, were not involved in LPS+LL-37 induced CXCL8 secretion. Expression of MD-2 was negligible in intestinal epithelium<sup>143, 193</sup> and only overexpression of MD-2 in HT29 cells promoted CXCL8 secretion upon LPS challenge<sup>194</sup>. Similarly, exogenous LBP failed to induce CXCL8 secretion in methotrexate differentiated HT29 cells challenged with LPS<sup>195</sup>. Therefore, we inferred that LPS+LL-37 physically interacts with TLR4 to promote colonic CXCL8 secretion, independent of MD-2 and LBP adaptors.

Intriguingly, the synergistic LPS+LL-37 effect on CXCL8 synthesis from the colonic epithelium was restricted to TLR4 signalling: LL-37 did not enhance CXCL8 production evoked by flagellin (TLR5 ligand) and actually reduced CXCL8 production elicited by TLR9 activation with ODN. Similar differential responses of cathelicidin to TLR ligands have been reported. In human bronchial epithelial cells, LL-37 increased CXCL8 secretion in the presence of the TLR1/2 ligand PAM3CSK4<sup>196</sup>, but decreased impact of dsRNAs (TLR3 ligand)<sup>196, 197</sup>. Also, *CXCL8* mRNA synthesis was reduced by LL-37+ODN in colonic epithelial cells<sup>163</sup>. That CXCL8 synthesis was still partially evident in TLR4 KD HT29 cells may reflect involvement of other PRRs and therefore needs further investigation. In conclusion, the outcome of cathelicidin-bacterial ligand(s) interaction appeared to be dependent on the nature of the bacterial molecule, microenvironmental conditions, the cell and the receptor (sub)types it expresses.

We concluded that two signal pathways converged on regulation of CXCL8 production by LPS+LL-37 in the colonic epithelium (**Fig 4.1**). First, LPS+LL-37 promoted *CXCL8* gene stability via p38MAPK activation, dependent on Src and EGFR-kinase activity.



Figure 4.1. Proposed signalling mechanisms elicited by cathelicidin in synergy with LPS to promote CXCL8 secretion in colonic epithelium and subsequent neutrophil recruitment/activation. In colonic epithelial cells, LL-37 physically bound and facilitated LPS uptake via interaction with GM1 in lipid rafts. Intracellularly, LPS interacted with TLR4 to promote activation of two signalling axes: one NF- $\kappa$ B dependent, which signals via MEK1/2, and another NF- $\kappa$ B independent, which relies on Src and EGFR kinases cross-talk and subsequent p38MAPK activation. Whereas NF- $\kappa$ B dependent signalling primarily regulated *CXCL8* mRNA synthesis, NF- $\kappa$ B independent signalling primarily controlled *CXCL8* mRNA stability. Both signalling pathways together promoted colonic CXCL8 protein synthesis and secretion. Induced colonic CXCL8 chemokine promoted intestinal defenses via neutrophil recruitment/activation.

Interestingly, p38MAPK extended *CXCL8* mRNA half-life in HT29 cells stimulated with TNF- $\alpha^{198}$ , perhaps by preventing deadenylation of *CXCL8* mRNA via interaction with AU rich-elements in 3'-untranslated regions of *CXCL8* gene<sup>199, 200</sup>. Thus, our reported Src-EGFR-p38MAPK signalling is a cathelicidin downstream effect in regulation of colonic CXCL8 synthesis, by aiding in mRNA stabilization. Second, CXCL8 synthesis was dependent on miR-31, MEK1/2 and NF- $\kappa$ B (p65) phosphorylation/activation. Likewise, LL-37 promoted NF- $\kappa$ B dependent CXCL8 secretion in human monocytes<sup>97</sup>. Similar to previous studies<sup>186</sup>, we determined *Camp*<sup>+/+</sup> BMMs had higher NF- $\kappa$ B activation than *Camp*<sup>-/-</sup> BMMs upon LPS challenge.

In summary, cathelicidin in concert with LPS evoked production and secretion of chemoattractant CXCL8 (humans)/ CXCL1 (mice) by colonic epithelium via ganglioside GM1/ lipid rafts mediated LPS internalization and sequential cross-talk with intracellular TLR4. This epithelial cathelicidin-mediated "pathogen sensing" of bacterial LPS, resulting in enhanced synthesis of a neutrophil chemokine CXCL8, represented an endogenous innate defense mechanism that triggers neutrophil migration into inflamed tissue in infectious colitis. This endogenous innate defence role of cathelicidin can be added to its already well-recognized antimicrobial properties.

# 4.2. Cathelicidin induced Tollip synthesis and prevented TLR-dependent apoptosis in colonic epithelium.

This study demonstrated that immunomodulatory concentrations of cathelicidin (< 4  $\mu$ M) reduced colonic epithelial apoptosis, invoked either by a combination of TNF- $\alpha$  and IFN- $\gamma$  or by infecting *Camp*<sup>+/+</sup> and *Camp*<sup>-/-</sup> mice with *C. rodentium* as a model of infectious colitis. This effect of cathelicidin seemed to be dependent on synthesis of a negative regulator of TLR/IRAK-1 signalling, called Tollip. Enteric bacterial infections are characterized by increased TLR signalling and aberrant colonic epithelial apoptosis that contribute to pathology<sup>201</sup>. In this regard, murine *C. rodentium* infection increases TLR signalling (MyD88)<sup>202</sup> and exaggerate apoptosis in colonic epithelium of C57BL/6 mice<sup>184, 203</sup>. Likewise, systemic administration of the bacterial virulence factor LPS (0.125 µg/g) induces significant apoptosis in villus epithelial cells as early as 1.5 h after inoculation<sup>204</sup>. Such apoptotic effects of colitic bacteria, or their associated factors, on epithelial cells could be partly attributed to the presence of pro-inflammatory cytokines, namely TNF- $\alpha$  and IFN- $\gamma$ <sup>205-207</sup>. Herein, cathelicidin reduced apoptosis in HT29 cells under the influence of TNF- $\alpha$  and IFN- $\gamma$  treatments. *In vivo, C. rodentium* infected *Camp*<sup>+/+</sup> mice had increased epithelial

apoptosis in the colon as compared to corresponding Camp<sup>-/-</sup> mice. Further, this antiapoptotic effect of cathelicidin seemed to be regulated by Tollip protein; HT29 cells deficient in Tollip expression were not protected by cathelicidin against activation of apoptotic markers, e.g. cleaved caspase-3 and cleaved PARP, by TNF-α and IFN-γ. Consistent with our findings, in previous studies, cathelicidin were anti-apoptotic at physiological concentrations (up to 4  $\mu$ M) but pro-apoptotic at higher concentrations (> 4  $\mu$ M). For instance, LL-37 (~ 0.2 µM) prevented LPS-induced apoptosis in primary endothelial cells and spontaneous apoptosis in human neutrophils<sup>64</sup>. Conversely, LL-37 (~ 20  $\mu$ M) induced caspase-independent apoptosis on colonic cancer cell lines<sup>68</sup> and LL-37 (> 4  $\mu$ M) induced apoptosis of unstimulated isolated murine CD8+ cells<sup>208</sup>. Likewise, Tollip differentially regulates apoptosis, depending on cell/tissue type. Tollip reduced DSS<sup>209</sup> and cytokine (TNF- $\alpha$  and IFN- $\gamma$ )<sup>60</sup> induced apoptosis in intestinal epithelium<sup>60, 209</sup>, but exacerbated injury in heart and brain tissues from myocardial infarction and intra-cerebral hemorrhage patients, respectively<sup>210, 211</sup>. Taken together, cathelicidin stimulate anti-apoptotic effects when present at physiological or endogenous concentrations; however, these effects seemed to rely on availability of Tollip protein.

Whereas effects of cathelicidin in inducing Tollip has been reported in various cell types (including colonic epithelium, fibroblasts and macrophages), cathelicidin enhanced Tollip protein synthesis, but not mRNA transcription, in colonic epithelium. Uncoupling of Tollip mRNA and protein expression has been reported in intestinal epithelium; Tollip protein concentrations were higher in large versus small intestine of C57BL/6 mice, although both types of intestines had similar levels of mRNA expression<sup>180</sup>. Furthermore, LL-37 induced Tollip protein synthesis had a bell-shaped curve, with maximum Tollip synthesis at relatively low concentrations of LL-37 (i.e., ~ 2  $\mu$ M). This is in agreement with with our 'Study 1'

87

wherein cathelicidin up to 4  $\mu$ M failed to induce any significant pro-inflammatory CXCL8 synthesis. Likewise, LL-37 only at relatively high concentration (~ 20  $\mu$ M) promoted CCL-2, CCL5 and CXCL10 in murine (RAW264.7) macrophages<sup>129</sup>.

IRAK-1 is a TLR adaptor protein which contributes immensely to expression of proinflammatory mediators in intestinal epithelium<sup>212, 213</sup>. Cathelicidin-induced production of Tollip prevented IRAK-1 phosphorylation and pro-inflammatory cytokine (IFN- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$ ) synthesis in colonic epithelium. The link between Tollip and IRAK-1 was reported in embryonic kidney (HEK293) epithelial cells, where Tollip overexpression prevented LPS mediated IRAK-1 phosphorylation<sup>178</sup> whereas reduced Tollip expression increased LPS mediated IL-6, TNF- $\alpha$  and IFN- $\beta$  secretion by murine macrophages<sup>116</sup>. In addition, induction of Tollip by epigallocatechin-3-gallate (a green tea component) prevented LPS mediated COX-2 and CXCL8 production in HT29 cells<sup>214</sup>. Overall, physiological amounts of cathelicidin maintain Tollip synthesis in colonic epithelium, perhaps as a regulatory mechanism to prevent unwarranted gut inflammation.

Initially known as microbicidal peptides, cathelicidin have been increasingly reported as potent immunomodulatory molecules. Cathelicidin interact, directly or indirectly, with various receptors, including EGFR<sup>179</sup>, P2X7<sup>81</sup> and FPR 2<sup>72</sup>. Here, cathelicidin induced Tollip synthesis in colonic epithelium was dependent on EGFR signalling. Consistent with this, in our 'Study 1,' LL-37/LPS promoted EGFR phosphorylation leading to enhanced production of the chemokine CXCL8 in colonic epithelium. Although it is not fully understood how EGFR regulates Tollip synthesis, EGF could modulate Tollip protein either through direct phosphorylation<sup>215</sup> or indirectly, by phosphorylating a member of Tom1L1 protein family that interacts with Tollip<sup>216, 217</sup>. One novel mechanism proposed on the basis of our 'Study 1',

is the role of cathelicidin in downregulating miR-31 expression in cell lysates of colonic epithelium. In recent studies, an LL-37 analogue (FF/CAP18) enhanced secretion of exosomes containing miR-31 from human colonic epithelial (HCT116) cells<sup>218</sup>, perhaps accounting for decreased intracellular miR-31 levels as observed in both 'Study 1' and the current study. Moreover, LL-37 and microRNAs reciprocally regulate expression of eachother for instance, LL-37 increased miR200c-3p levels in HT29 cells; conversely, overexpression of miR-130a by pEGP-miR-130a plasmid transfection into murine BMMs reduced expression of cathelicidin Camp gene<sup>218-220</sup>. Here, cathelicidin reduced colonic miR-31 was dependent on EGFR activation. This new mechanism (cathelicidin negatively regulates miRNA via EGFR) would explain previous studies in which reduced miR-31 levels were associated with enhanced Tollip expression in large intestine of C57BL/6 mice<sup>180</sup>, whereas EGFR signalling down-regulated expression of miR-143 and miR-145 in colonic tumours<sup>221</sup>, but up-regulated miR-21 in lung cancer biopsies and immortalized human bronchial epithelial (HBET2) cells<sup>222</sup>. EGFR mediated miR-31 down-regulation was dependent on increased phosphorylation of AGO2, a dicer protein involved in maturation of micro RNAs<sup>223, 224</sup>. In agreement, cervical HeLa cells under hypoxia had EGF phosphorylated AGO2 via EGFR and reduced miR-31 levels<sup>225</sup>. Furthermore, EGFR enhanced only AGO2, and not AGO1, AGO3 or AGO4 protein stability in breast cancer cell lines<sup>226</sup>. EGFR bound miRNA-mRNA complexes in nucleus and thereby, regulated mRNA processing/protein stability of genes downstream of vascular endothelial growth factor (VEGF)/HIF-1A signalling pathway in human alveolar epithelial (A549) cells<sup>227</sup>. As colonic surface epithelium is homeostatically maintained in hypoxic environment, the cathelicidin/EGFR/AGO2/miR-31 dependent Tollip regulation could be tissue-gut specific.
In conclusion, cathelicidin maintained levels of Tollip and prevent cell apoptosis at colonic mucosa, a process that could prevent exaggerated and chronic inflammation under physiological or infectious conditions. Understanding mechanistic relevance of concentration-function(s) of cathelicidin peptide will help in development of better therapeutics against colitis.

#### **CHAPTER 5 - CONCLUSIONS AND FUTURE DIRECTION**

Cathelicidin are multifunctional peptides with diverse functions, including microbicidal, immunomodulatory, anti- and pro-apoptotic effects, as well as ability to promote wound healing and angiogenesis. Those immunomodulatory functions can regulate both pro- and anti-inflammatory processes. Mechanisms elicited by these pleiotropic peptides are not fully elucidated, but appear to depend on type of cell studied (hematopoietic versus nonhematopoietic), micro-environment (surrounding cytokine milieu), type of stimulus (sterile or infectious), concentration of cathelicidin at the site and presence or absence of inhibitors (e.g. salt or serum). Although cathelicidin and other HDPs have been proposed for immune-based therapies, it is essential to decipher their mechanisms of action to minimize off-target effects. This thesis explored novel functions of human cathelicidin LL-37 (*in vitro*) and murine CRAMP (*in vivo/ex vivo*) in the colon that regulate physiological homeostasis and responses to enteric bacterial infections. Although LL-37 activity was assessed in murine colonic epithelium, further work is needed to validate cross-species reactivity of LL-37 and CRAMP. Thus, it would be premature to extrapolate findings of this thesis to the role of other cathelicidins in gut immunity.

In the first study, the role of cathelicidin in regulating TLR synthesis and function was investigated. Under physiological conditions, colonic epithelium represents an immunologically hyporesponsive site to LPS challenge, a beneficial strategy to avoid unwanted inflammation and hence, chronic diseases (e.g. IBDs). However, it is incompletely understood how colonic epithelium can switch to become an immunologically responsive site and mount an innate response to clear pathogens (e.g. Gram negative bacterial infection). Based on present results, cathelicidin and LPS, in combination but not individually, initiated immunological signalling from colonic epithelium and enhanced expression of the neutrophil

chemoattractant CXCL8/CXCL1. In addition, I further identified a unique two-signal axis only triggered by a complex of cathelicidin and LPS in vitro. This increased signalling was preceded by an increased uptake of LPS and cathelicidin by an otherwise hyporesponsive colonic epithelium. Whereas my work indicated that cathelicidin contributed to CXCL1 production and neutrophil recruitment, there are certain inherent limitations that need to be addressed in future studies. First, in this study, we have not unequivocally established if CXCL1 or cathelicidin through one of its known receptors (e.g., FPR2) contributes to neutrophil recruitment and activation upon C. rodentium infection in mice. This can be tested by providing exogenous cathelicidin separately to CXCL1 and/or CXCR1/2 deficient mice, followed by infection with C. rodentium. Second, we co-related reduced fecal shedding of C. rodentium in infected wild type mice to increased recruitment of neutrophils; however, this hypothesis needs to be further validated. Using bone marrow transfers (cathelicidin null to wild type), it should be determined whether hematopoietic or non-hematopoietic cells contribute to C. rodentium killing in wild type infected mice. Intrinsic defects in neutrophil killing ability should be tested by incubating C. rodentium with isolated neutrophils from wild type and cathelicidin-deficient mice. Further, to ascertain the role of neutrophils in C. *rodentium* killing, neutrophil neutralization should be done with anti-Ly6G (anti-Gr1) antibody and then fecal shedding of C. rodentium must be compared to corresponding wild type infected mice. In the present study, S. Typhimurium synergized with cathelicidin to enhance CXCL8 synthesis; however, whether cathelicidin enhances neutrophil recruitment and S. Typhimurium killing in wild type verus cathelicidin null mice was not determined in *vivo*. This should be done to validate importance of cathelicidin in promoting neutrophil recruitment and conferring protection against common diarrhea causing pathogens in humans. Fourth, although this study was focused on neutrophils, cathelicidin differentially regulate expression of various chemokines in C. rodentium infected mice. Role of

cathelicidin in recruitment of other immune cell populations should be determined. For example, CXCL9 and CXCL10 promote NK cell recruitment to the infected site. Fifth, in this study, cathelicidin promoted LPS uptake via GM1 lipid rafts in colonic epithelium. However, why GM1 is the preferred ganglioside was not explored mechanistically. Given the importance of GM1 in cholera disease is a strong impetus to delineate how cathelicidin specifically interacts with GM1, perhaps using a mix of bio-informatics and molecular tools. Sixth, we proposed an elegant mechanism by which LPS signals via intracellular TLR4 in colonic epithelium. To validate this hypothesis, confocal microscopy should be used to visualize intracellular co-localization of TLR4 and LPS. Further, to determine if surface TLR4 are involved in LPS-LL-37 synergism, TLR4 KD cells should be tested for defects in LPS uptake in presence of cathelicidin. Seventh, although our study indicated that additional receptor(s) other than TLR4 could be involved in LPS-LL-37 induced CXCL8 synthesis, this hypothesis was not tested. One potential avenue for future study is to determine the role of casapase-11<sup>228</sup>, an intracellular LPS receptor. Caspase-11 KD colonic epithelial cells should be constructed, followed by evaluation of CXCL8 secretion upon LPS-LL-37 treatment. Eighth, in this study, cathelicidin promoted CXCL8 stability via EGFR-Src-p38MAPK axis. This finding needs further validation by additional experiments. Whether direct inhibition of EGFR and/or Src kinase regulate(s) CXCL8 gene stability should be evaluated in colonic epithelial cells. Ninth, we elucidated a novel signalling mechanism regulating chemokine expression by colonic epithelium in vitro. Future studies should involved monitoring molecular pathways in more physiologically relevant models, e.g. 3D organoid culture and murine models. Although this study highlighted a potential role of cathelicidin as an immunomodulatory antimicrobial, targeted delivery of the peptide poses a serious challenge. Various delivery strategies have been investigated in animal-based models including: Eudragit FS30D polymer-coated CSA13 peptide (dissolves only in basic pH, thereby

targeting the intestine) delivery in colon<sup>229</sup>; nanofiber encapsulation of cathelicidin peptide for sustained delivery into chronic skin wounds<sup>230</sup>; and genetically engineered commensals (*Lactococcus lactis*) over-expressing cathelicidin delivery into colon via oral inoculation<sup>231</sup>. Future studies could explore these delivery strategies to confirm effects of cathelicidin and their efficiency in controlling enteric infections.

The role of cathelicidin in a normal physiological setting in the gut has been poorly studied. The gut is of particular importance, as colonic mucosa is normally hyporesponsive. In Study 2, a new role of cathelicidin was proposed, whereby it maintains homeostasis of colonic epithelium through synthesis of Tollip, a key negative regulator of TLR signalling. Induced Tollip expression has been reported in other cell types, e.g. murine BMMs and murine fibroblasts. However, role of Tollip in preventing TLR-dependent apoptosis, either by a mixture of pro-inflammatory cytokines or in response to enteric bacterial infection, has only been evaluated in colonic epithelium. This proof of concept provided a basis for future experiments involving Tollip-deficient animals treated with synthetic cathelicidin and challenged with enteric bacteria. Such studies may confirm that cathelicidin facilitates antiapoptotic function via Tollip.

My work provided evidence that cathelicidin induces Tollip synthesis by promoting post translational stabilization of Tollip protein and thereby prevents apoptosis in colonic epithelium. Some limitations of this study are: first, this study did not evaluate whether cathelicidin specifically induced Tollip synthesis or it could regulate other TLR inhibitors, namely SIGIRR and IRAK-M. Testing pan-TLR inhibitors will help to characterize role of cathelicidin in modulation of intestinal hyporesponsiveness. Second, although that miR-31 was involved in post-translation stabilization of Tollip, a negative miRNA control is required to unambiguously support our conclusions. Further, to re-assure role of cathelicidin in Tollip stability, Tollip protein turnover under homeostatic (unstimulated) conditions should be determined in cathelicidin-deficient colonic epithelial cells and compared to sham-controls. Third, the mechanism of cathelicidin mediated Tollip synthesis such as miR-31 degradation via an EGFR-AGO2, were not evaluated in primary cell cultures thus, does not necessarily represent an in vivo phenomenon. To address such concerns, these mechanism should be verified in vivo in miR-31 over-expressing mice or in ex vivo settings using miR-31 overexpressing organoid cultures treated with cathelicidin. Additionally, 3D organoids obtained from EGFR deficient mice, treated or not with exogenous cathelicidin, could be used to quantify miRNA-31 levels and Tollip protein availability. The epigenetic role of cathelicidin in regulation of miRNA maturation and processing, opens new avenues of research to determine how and which miRNAs are regulated by cathelicidin and their impacts on infectious diseases. Global miRNA expression profile under influence of cathelicidin in presence or not of enteric bacteria infection, should be studied to identify novel functional targets for cathelicidin. Such cathelicidin-dependent miRNA regulation studies at colonic mucosa will assist by recognizing new inhibitor-based therapeutics for gut inflammatory diseases and, in determining off-target(s) effects of cathelicidin. Fourth, the apoptosis in C. rodentium infected cathelicidin null mice is attributed to Tollip protein deficiency. However, it could perhaps be an outcome of increased C. rodentium burden as shown in Study 1. This hypothesis can be tested by co-relating fecal shedding of bacteria and colonic epithelial apoptosis at various intervals post C. rodentium challenge (days 3, 5, 7 and 10) in cathelicidin-deficient mice. Further, C. rodentium induced apoptosis should be assessed in cathelicidin-Tollip double deficient mice after exogenous supplementation with cathelicidin. Determination of apoptosis in such genetically ablated mice with proper controls would provide a clear answer regarding whether cathelicidin prevents apoptosis through Tollip.

On the basis of this thesis work, I concluded cathelicidin "fine tunes" the immune response at the colonic mucosa. In sterile, homeostatic conditions, cathelicidin at a physiological concentration (~  $2-4 \mu$ M) can help colonic epithelium maintain relatively hyporesponsive/anti-inflammatory state by inducing expression of TLR negative regulators such as Tollip. Furthermore, under diseased conditions, cathelicidin can act as a pro-inflammatory factor by inducing secretion of chemokines by colonic epithelium and thus, promoting granulocyte recruitment and activation, via combination with various virulent factors e.g. LPS. This targeted pro-inflammatory response induced by cathelicidin during an enteric infection could help clear the pathogen and thus, ameliorate disease-induced pathology.

#### REFERENCES

- Navaneethan U, Giannella RA. Infectious colitis. Current Opinion in Gastroenterology 2011;27:66-71.
- 2. Cherubin CE, Fodor T, Denmark LI, et al. Symptoms, septicemia and death in salmonellosis. American Journal of Epidemiology 1969;90:285-91.
- Wilcock BP. Experimental Klebsiella and Salmonella infection in neonatal swine. Canadian Journal of Comparative Medicine 1979;43:200-6.
- Farthing MJG. Infectious colitis. In Inflammatory Bowel Disease: From Bench to Bedside, (Eds Targan S. et al.) New York: Springer 2003:845–861
- 5. Brunelle BW, Bearson BL, Bearson SMD, et al. Multidrug-resistant *Salmonella enterica* serovar Typhimurium isolates are resistant to antibiotics that influence their swimming and swarming motility. mSphere 2017;2.
- Odonkor ST, Addo KK. Prevalence of multidrug-resistant *Escherichia coli* isolated from drinking water sources. International Journal of Microbiology 2018;2018:7204013.
- Neuman H, Forsythe P, Uzan A, et al. Antibiotics in early life: dysbiosis and the damage done. FEMS Microbiology Reviews 2018;42:489-499.
- Sarker P, Mily A, Mamun AA, et al. Ciprofloxacin affects host cells by suppressing expression of the endogenous antimicrobial peptides cathelicidins and beta-defensin-3 in colon epithelia. Antibiotics (Basel) 2014;3:353-74.
- 9. Cross AS. What is a virulence factor? Critical Care 2008;12:196.
- Ramachandran G. Gram-positive and gram-negative bacterial toxins in sepsis: a brief review. Virulence 2014;5:213-8.
- Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annual Review of Biochemistry 2002;71:635-700.

- Gorelik A, Illes K, Nagar B. Crystal structure of the mammalian lipopolysaccharide detoxifier. Proceedings of the National Academy of Sciences of the United States of America 2018;115:E896-E905.
- 13. Rhee SH. Lipopolysaccharide: basic biochemistry, intracellular signaling, and physiological impacts in the gut. Intestinal Research 2014;12:90-5.
- 14. Bashir A. BZM, Haq E. Lipopolysaccharide, mediator of sepsis enigma: recognition and signaling. Internatonal Journal of Biochemistry Research & Review 2011;1:1-13.
- 15. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine 2008;42:145-151.
- Murdock JL, Nunez G. TLR4: The winding road to the discovery of the LPS receptor. Journal of Immunology 2016;197:2561-2.
- Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes.
   Experimental & Molecular Medicine 2013;45:e66.
- Allaire JM, Crowley SM, Law HT, et al. The intestinal epithelium: Central coordinator of mucosal immunity. Trends in Immunology 2018;39:677-696.
- Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nature Reviews. Immunology 2014;14:667-85.
- Lipkin M, Bell B, Sherlock P. Cell proliferation kinetics in the gastrointestinal tract of man. I. Cell renewal in colon and rectum. The Journal of Clinical Investigation 1963;42:767-76.
- Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nature Reviews. Immunology 2003;3:331-41.
- Turvey SE, Broide DH. Innate immunity. The Journal of Allergy and Clinical Immunology 2010;125:S24-32.

- 23. Kosciuczuk EM, Lisowski P, Jarczak J, et al. Cathelicidins: family of antimicrobial peptides. A review. Molecular Biology Reports 2012;39:10957-70.
- 24. Hancock RE, Haney EF, Gill EE. The immunology of host defence peptides: beyond antimicrobial activity. Nature Reviews Immunology 2016;16:321-34.
- 25. van Harten RM, van Woudenbergh E, van Dijk A, et al. Cathelicidins: Immunomodulatory antimicrobials. Vaccines (Basel) 2018;6.
- 26. Zhao C, Ganz T, Lehrer RI. Structures of genes for two cathelin-associated antimicrobial peptides: prophenin-2 and PR-39. FEBS Letters 1995;376:130-4.
- Termen S, Tollin M, Rodriguez E, et al. PU.1 and bacterial metabolites regulate the human gene *CAMP* encoding antimicrobial peptide LL-37 in colon epithelial cells. Molecular Immunology 2008;45:3947-55.
- Xia C, Cheshire JK, Patel H, et al. Cross-talk between transcription factors NF-kappa
   B and C/EBP in the transcriptional regulation of genes. The International Journal of
   Biochemistry & Cell Biology 1997;29:1525-39.
- Huttner KM, Bevins CL. Antimicrobial peptides as mediators of epithelial host defense.
   Pediatric Research 1999;45:785-94.
- 30. Scheb-Wetzel M, Rohde M, Bravo A, et al. New insights into the antimicrobial effect of mast cells against *Enterococcus faecalis*. Infection and Immunity 2014;82:4496-507.
- Xia X, Zhang L, Wang Y. The antimicrobial peptide cathelicidin-BF could be a potential therapeutic for *Salmonella* Typhimurium infection. Microbiological Research 2015;171:45-51.
- Scheenstra MR, van den Belt M, Tjeerdsma-van Bokhoven JLM, et al. Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action. Scientific Reports 2019;9:4780.

- 33. Wang J, Wong ES, Whitley JC, et al. Ancient antimicrobial peptides kill antibioticresistant pathogens: Australian mammals provide new options. PLoS One 2011;6:e24030.
- 34. Braff MH, Jones AL, Skerrett SJ, et al. *Staphylococcus aureus* exploits cathelicidin antimicrobial peptides produced during early pneumonia to promote staphylokinase-dependent fibrinolysis. Journal of Infectious Diseases 2007;195:1365-72.
- 35. Lee PH, Ohtake T, Zaiou M, et al. Expression of an additional cathelicidin antimicrobial peptide protects against bacterial skin infection. Proceedings of the National Academy of Sciences of The United States Of America 2005;102:3750-5.
- Boman HG, Agerberth B, Boman A. Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. Infection and Immunity 1993;61:2978-84.
- 37. Shi J, Ross CR, Chengappa MM, et al. Antibacterial activity of a synthetic peptide (PR-26) derived from PR-39, a proline-arginine-rich neutrophil antimicrobial peptide.
  Antimicrobial Agents and Chemotherapy 1996;40:115-21.
- 38. Kanthawong S, Bolscher JG, Veerman EC, et al. Antimicrobial and antibiofilm activity of LL-37 and its truncated variants against *Burkholderia pseudomallei*. International Journal of Antimicrobial Agents 2012;39:39-44.
- 39. Cuperus T, van Dijk A, Matthijs MG, et al. Protective effect of in ovo treatment with the chicken cathelicidin analog D-CATH-2 against avian pathogenic E. coli. Scientific Reports 2016;6:26622.
- 40. Johansson J, Gudmundsson GH, Rottenberg ME, et al. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. The Journal of Biological Chemistry 1998;273:3718-24.

- Wang YH, Xia JL, Wang WM, et al. TNFalpha induced IL-8 production through p38 MAPK- NF-kB pathway in human hepatocellular carcinoma cells. Chinese Journal of Hepatology 2011;19:912-6.
- 42. Huang LC, Jean D, McDermott AM. Effect of preservative-free artificial tears on the antimicrobial activity of human beta-defensin-2 and cathelicidin LL-37 in vitro. Eye Contact Lens 2005;31:34-8.
- 43. Ciornei CD, Sigurdardottir T, Schmidtchen A, et al. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrobial Agents and Chemotherapy 2005;49:2845-50.
- Zanetti M. The role of cathelicidins in the innate host defenses of mammals. Current Issues in Intestinal Microbiology 2005;7:179-96.
- 45. Jorgensen I, Rayamajhi M, Miao EA. Programmed cell death as a defence against infection. Nature Reviews. Immunology 2017;17:151-164.
- 46. Labbe K, Saleh M. Cell death in the host response to infection. Cell death and Differentiation 2008;15:1339-49.
- 47. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic Pathology 2007;35:495-516.
- 48. Fernandez MI, Regnault B, Mulet C, et al. Maturation of paneth cells induces the refractory state of newborn mice to Shigella infection. Journal of Immunology 2008;180:4924-30.
- 49. Bucker R, Schulz E, Gunzel D, et al. Alpha-Haemolysin of *Escherichia coli* in IBD: a potentiator of inflammatory activity in the colon. Gut 2014;63:1893-901.
- 50. Torchinsky MB, Garaude J, Martin AP, et al. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. Nature 2009;458:78-82.

- Di Sabatino A, Ciccocioppo R, Luinetti O, et al. Increased enterocyte apoptosis in inflamed areas of Crohn's disease. Diseases of the Colon and Rectum 2003;46:1498-507.
- 52. Ramachandran A, Madesh M, Balasubramanian KA. Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions. Journal of Gastroenterology and Hepatology 2000;15:109-20.
- 53. Iwamoto M, Koji T, Makiyama K, et al. Apoptosis of crypt epithelial cells in ulcerative colitis. The Journal of Pathology 1996;180:152-9.
- 54. Parker A, Vaux L, Patterson AM, et al. Elevated apoptosis impairs epithelial cell turnover and shortens villi in TNF-driven intestinal inflammation. Cell Death & Disease 2019;10:108.
- 55. Zychlinsky A, Prevost MC, Sansonetti PJ. *Shigella flexneri* induces apoptosis in infected macrophages. Nature 1992;358:167-9.
- 56. Hersh D, Monack DM, Smith MR, et al. The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. Proceedings of the National Academy of Sciences of the United States of America 1999;96:2396-401.
- 57. Denecker G, Declercq W, Geuijen CA, et al. Yersinia enterocolitica YopP-induced apoptosis of macrophages involves the apoptotic signaling cascade upstream of bid. The Journal of Biological Chemistry 2001;276:19706-14.
- 58. Jung HC, Eckmann L, Yang SK, et al. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. The Journal of Clinical Investigation 1995;95:55-65.
- 59. Waki N, Kuwabara Y, Yoshikawa Y, et al. Amelioration of *Citrobacter rodentium* proliferation in early stage of infection in mice by pretreatment with *Lactobacillus*

*brevis* KB290 and verification using in vivo bioluminescence imaging. FEMS Microbiology Letters 2017;364.

- 60. Mukherjee S, Biswas T. Activation of TOLLIP by porin prevents TLR2-associated IFN-gamma and TNF-alpha-induced apoptosis of intestinal epithelial cells. Cell Signal 2014;26:2674-82.
- 61. Marini M, Bamias G, Rivera-Nieves J, et al. TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. Proceedings of the National Academy of Sciences of the United States of America 2003;100:8366-71.
- 62. Fries W, Muja C, Crisafulli C, et al. Infliximab and etanercept are equally effective in reducing enterocyte APOPTOSIS in experimental colitis. International Journal of Medical Sciences 2008;5:169-80.
- 63. Schaller-Bals S, Schulze A, Bals R. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. American Journal of Respiratory and Critical Care Medicine 2002;165:992-5.
- Suzuki K, Murakami T, Kuwahara-Arai K, et al. Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells. International Immunology 2011;23:185-93.
- 65. Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. J Immunol 2006;176:3044-52.
- 66. Wu J, Parungo C, Wu G, et al. PR39 inhibits apoptosis in hypoxic endothelial cells: role of inhibitor apoptosis protein-2. Circulation 2004;109:1660-7.

- 67. Barlow PG, Beaumont PE, Cosseau C, et al. The human cathelicidin LL-37 preferentially promotes apoptosis of infected airway epithelium. Americal Journal of Respiratory Cell and Molecular Biology 2010;43:692-702.
- 68. Ren SX, Cheng AS, To KF, et al. Host immune defense peptide LL-37 activates caspase-independent apoptosis and suppresses colon cancer. Cancer Research 2012;72:6512-23.
- 69. Wong JH, Ye XJ, Ng TB. Cathelicidins: peptides with antimicrobial, immunomodulatory, anti-inflammatory, angiogenic, anticancer and procancer activities. Current Protein & Peptide Science 2013;14:504-14.
- 70. Tjabringa GS, Aarbiou J, Ninaber DK, et al. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. Journal of Immunology 2003;171:6690-6.
- 71. Girnita A, Zheng H, Gronberg A, et al. Identification of the cathelicidin peptide LL-37 as agonist for the type I insulin-like growth factor receptor. Oncogene 2012;31:352-65.
- 72. De Y, Chen Q, Schmidt AP, et al. LL-37, the neutrophil granule- and epithelial cellderived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. Journal of Experimental Medicine 2000;192:1069-74.
- 73. Zhang Z, Cherryholmes G, Chang F, et al. Evidence that cathelicidin peptide LL-37 may act as a functional ligand for CXCR2 on human neutrophils. European Journal of Immunology 2009;39:3181-94.
- 74. Subramanian H, Gupta K, Guo Q, et al. Mas-related gene X2 (MrgX2) is a novel G protein-coupled receptor for the antimicrobial peptide LL-37 in human mast cells: resistance to receptor phosphorylation, desensitization, and internalization. The Journal of Biological Chemistry 2011;286:44739-49.

- 75. Brandenburg LO, Jansen S, Wruck CJ, et al. Antimicrobial peptide rCRAMP induced glial cell activation through P2Y receptor signalling pathways. Molecular Immunology 2010;47:1905-13.
- 76. Mookherjee N, Lippert DN, Hamill P, et al. Intracellular receptor for human host defense peptide LL-37 in monocytes. Journal of Immunology 2009;183:2688-96.
- Tjabringa GS, Ninaber DK, Drijfhout JW, et al. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors.
   International Archives of Allergy and Immunology 2006;140:103-12.
- Rehman A, Sina C, Gavrilova O, et al. Nod2 is essential for temporal development of intestinal microbial communities. Gut 2011;60:1354-62.
- 79. Kurosaka K, Chen Q, Yarovinsky F, et al. 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. Journal of Immunology 2005;174:6257-65.
- 80. Niyonsaba F, Iwabuchi K, Someya A, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. Immunology 2002;106:20-6.
- 81. Montreekachon P, Chotjumlong P, Bolscher JG, et al. Involvement of P2X(7) purinergic receptor and MEK1/2 in interleukin-8 up-regulation by LL-37 in human gingival fibroblasts. Journal of Periodontal Research 2011;46:327-37.
- Wu WK, Wong CC, Li ZJ, et al. Cathelicidins in inflammation and tissue repair: Potential therapeutic applications for gastrointestinal disorders. Acta Pharmacologica Sinica 2010;31:1118-22.
- 83. Bowdish DM, Davidson DJ, Speert DP, et al. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. Journal of Immunology 2004;172:3758-65.

- 84. Scott MG, Davidson DJ, Gold MR, et al. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. Journal of Immunology 2002;169:3883-91.
- 85. Yu J, Mookherjee N, Wee K, et al. Host defense peptide LL-37, in synergy with inflammatory mediator IL-1beta, augments immune responses by multiple pathways. Journal of Immunology 2007;179:7684-91.
- 86. Shaykhiev R, Sierigk J, Herr C, et al. The antimicrobial peptide cathelicidin enhances activation of lung epithelial cells by LPS. Journal of Federation of American Societies for Experimental Biology 2010;24:4756-66.
- 87. McHugh BJ, Wang R, Li HN, et al. Cathelicidin is a "fire alarm", generating protective NLRP3-dependent airway epithelial cell inflammatory responses during infection with *Pseudomonas aeruginosa*. PLoS Pathogens 2019;15:e1007694.
- 88. van der Does AM, Beekhuizen H, Ravensbergen B, et al. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. Journal of Immunology 2010;185:1442-9.
- Bandholtz L, Ekman GJ, Vilhelmsson M, et al. Antimicrobial peptide LL-37 internalized by immature human dendritic cells alters their phenotype. Scandinavian Journal of Immunology 2006;63:410-9.
- 90. Lande R, Gregorio J, Facchinetti V, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 2007;449:564-9.
- 91. Davidson DJ, Currie AJ, Reid GS, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. Journal of Immunology 2004;172:1146-56.

- 92. Pistolic J, Cosseau C, Li Y, et al. Host defence peptide LL-37 induces IL-6 expression in human bronchial epithelial cells by activation of the NF-kappaB signaling pathway. Journal of Innate Immunology 2009;1:254-67.
- 93. Chen X, Takai T, Xie Y, et al. Human antimicrobial peptide LL-37 modulates proinflammatory responses induced by cytokine milieus and double-stranded RNA in human keratinocytes. Biochemical and Biophysical Research Communications 2013;433:532-7.
- 94. Nijnik A, Hancock RE. The roles of cathelicidin LL-37 in immune defences and novel clinical applications. Current Opinion in Hematology 2009;16:41-7.
- 95. Alalwani SM, Sierigk J, Herr C, et al. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. European Journal of Immunology 2010;40:1118-26.
- 96. Zughaier SM, Shafer WM, Stephens DS. Antimicrobial peptides and endotoxin inhibit cytokine and nitric oxide release but amplify respiratory burst response in human and murine macrophages. Cell Microbiology 2005;7:1251-62.
- 97. Mookherjee N, Hamill P, Gardy J, et al. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. Molecular BioSystems 2009;5:483-96.
- Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annual Review of Immunology 2002;20:197-216.
- 99. Kubarenko A, Frank M, Weber AN. Structure-function relationships of Toll-like receptor domains through homology modelling and molecular dynamics. Biochemical Society Transactions 2007;35:1515-8.

- 100. Sabah-Ozcan S, Baser A, Olcucu T, et al. Human TLR gene family members are differentially expressed in patients with urothelial carcinoma of the bladder. Urologic Oncology 2017.
- 101. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature Reviews. Immunology 2010;10:131-44.
- 102. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology 2004;126:1054-70.
- 103. Price AE, Shamardani K, Lugo KA, et al. A map of Toll-like receptor expression in the intestinal epithelium reveals distinct spatial, cell type-specific, and temporal patterns. Immunity 2018;49:560-575 e6.
- 104. Torok HP, Bellon V, Konrad A, et al. Functional Toll-Like Receptor (TLR)2 polymorphisms in the susceptibility to inflammatory bowel disease. PLoS One 2017;12:e0175180.
- 105. Caradonna L, Amati L, Magrone T, et al. Enteric bacteria, lipopolysaccharides and related cytokines in inflammatory bowel disease: biological and clinical significance. Journal of Endotoxin Research 2000;6:205-14.
- 106. Pedersen G, Andresen L, Matthiessen MW, et al. Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium. Clinical and Experimental Immunology 2005;141:298-306.
- 107. Koon HW, Shih DQ, Chen J, et al. Cathelicidin signaling via the toll-like receptor protects against colitis in mice. Gastroenterology 2011;141:1852-1863.
- 108. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infection and Immunity 2000;68:7010-7.

- 109. Marin M, Holani R, Shah CB, et al. Cathelicidin modulates synthesis of Toll-like receptors (TLRs) 4 and 9 in colonic epithelium. Molecular Immunology 2017;91:249-258.
- 110. Furrie E, Macfarlane S, Thomson G, et al. Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. Immunology 2005;115:565-74.
- 111. Kinnebrew MA, Ubeda C, Zenewicz LA, et al. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. The Journal of Infectious Diseases 2010;201:534-43.
- 112. Cario E, Brown D, McKee M, et al. Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. The American Journal of Pathology 2002;160:165-73.
- 113. Melmed G, Thomas LS, Lee N, et al. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. Journal of Immunology 2003;170:1406-15.
- 114. Shibolet O, Podolsky DK. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. American Journal of Physiology. Gastrointestinal and Liver Physiology 2007;292:G1469-73.
- 115. Burns K, Clatworthy J, Martin L, et al. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nature Cell Biology 2000;2:346-51.
- 116. Liu Y, Zhang Q, Ding Y, et al. Histone lysine methyltransferase Ezh1 promotes TLRtriggered inflammatory cytokine production by suppressing Tollip. Journal of Immunology 2015;194:2838-46.

- 117. Byun EB, Kim WS, Sung NY, et al. Epigallocatechin-3-gallate regulates antiinflammatory action through 67-kda laminin receptor-mediated Tollip signaling induction in lipopolysaccharide-stimulated human intestinal epithelial cells. Cellular Physiology and Biochemistry 2018;46:2072-2081.
- 118. Wald D, Qin J, Zhao Z, et al. SIGIRR, a negative regulator of Toll-like receptorinterleukin 1 receptor signaling. Nature Immunology 2003;4:920-7.
- 119. Garlanda C, Riva F, Polentarutti N, et al. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proceedings of the National Academy of Sciences of the United States of America 2004;101:3522-6.
- 120. Dubuquoy L, Bourdon C, Peuchmaur M, et al. Peroxisome proliferator-activated receptor (PPAR) gamma: a new target for the treatment of inflammatory bowel disease. Gastroenterologie Clinique et Biologique 2000;24:719-24.
- 121. Dubuquoy L, Jansson EA, Deeb S, et al. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. Gastroenterology 2003;124:1265-76.
- 122. Eun CS, Han DS, Lee SH, et al. Attenuation of colonic inflammation by PPARgamma in intestinal epithelial cells: effect on Toll-like receptor pathway. Digestive Diseases and Sciences 2006;51:693-7.
- 123. Yi H, Hu W, Chen S, et al. Cathelicidin-WA improves intestinal epithelial barrier function and enhances host defense against enterohemorrhagic *Escherichia coli* O157:H7 infection. Journal of Immunology 2017;198:1696-1705.
- 124. Yoo JH, Ho S, Tran DH, et al. Anti-fibrogenic effects of the anti-microbial peptide cathelicidin in murine colitis-associated fibrosis. Cellular and Molecular Gastroenterology and Hepatology 2015;1:55-74 e1.

- 125. Hing TC, Ho S, Shih DQ, et al. The antimicrobial peptide cathelicidin modulates *Clostridium difficile*-associated colitis and toxin A-mediated enteritis in mice. Gut 2013;62:1295-305.
- 126. Chromek M, Arvidsson I, Karpman D. The antimicrobial peptide cathelicidin protects mice from *Escherichia coli* O157:H7-mediated disease. PLoS One 2012;7:e46476.
- 127. Kao C, Lin X, Yi G, et al. Cathelicidin antimicrobial peptides with reduced activation of Toll-like receptor signaling have potent bactericidal activity against colistin-resistant bacteria. MBio 2016;7.
- 128. Bommineni YR, Dai H, Gong YX, et al. Fowlicidin-3 is an alpha-helical cationic host defense peptide with potent antibacterial and lipopolysaccharide-neutralizing activities. The FEBS Journal 2007;274:418-28.
- 129. Coorens M, Scheenstra MR, Veldhuizen EJ, et al. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. Scientific Reports 2017;7:40874.
- 130. Smith JJ, Travis SM, Greenberg EP, et al. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell 1996;85:229-36.
- Scott A, Weldon S, Buchanan PJ, et al. Evaluation of the ability of LL-37 to neutralise LPS in vitro and ex vivo. PLoS One 2011;6:e26525.
- Guo L, Lim KB, Poduje CM, et al. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 1998;95:189-98.
- 133. Sandgren S, Wittrup A, Cheng F, et al. The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. The Journal of Biological Chemistry 2004;279:17951-6.

- 134. Niyonsaba F, Ushio H, Hara M, et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. J Immunol 2010;184:3526-34.
- 135. Otte JM, Zdebik AE, Brand S, et al. Effects of the cathelicidin LL-37 on intestinal epithelial barrier integrity. Regulatory Peptides 2009;156:104-17.
- 136. Tokumaru S, Sayama K, Shirakata Y, et al. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. J Immunol 2005;175:4662-8.
- 137. Sturm A, Baumgart DC, d'Heureuse JH, et al. CXCL8 modulates human intestinal epithelial cells through a CXCR1 dependent pathway. Cytokine 2005;29:42-8.
- Maheshwari A, Lacson A, Lu W, et al. Interleukin-8/CXCL8 forms an autocrine loop in fetal intestinal mucosa. Pediatric Research 2004;56:240-9.
- 139. Tai EK, Wong HP, Lam EK, et al. Cathelicidin stimulates colonic mucus synthesis by up-regulating MUC1 and MUC2 expression through a mitogen-activated protein kinase pathway. Journal of Cellular Biochemistry 2008;104:251-8.
- 140. Cobo ER, Kissoon-Singh V, Moreau F, et al. Muc2 mucin and butyrate contribute to the synthesis of the antimicrobial peptide cathelicidin in response to *Entamoeba histolytica-* and dextran sodium sulfate-induced colitis. Infection and Immunity 2017.
- 141. Kolios G, Petoumenos C, Nakos A. Mediators of inflammation: production and implication in inflammatory bowel disease. Hepatogastroenterology 1998;45:1601-9.
- 142. Pallone F, Monteleone G. Regulatory cytokines in inflammatory bowel disease.Alimentary Pharmacology & Therapeutics 1996;10 Suppl 2:75-9; discussion 80.
- 143. Vamadevan AS, Fukata M, Arnold ET, et al. Regulation of Toll-like receptor 4associated MD-2 in intestinal epithelial cells: a comprehensive analysis. Innate Immunity 2010;16:93-103.

- 144. Redfern RL, Reins RY, McDermott AM. Toll-like receptor activation modulates antimicrobial peptide expression by ocular surface cells. Experimental Eye Research 2011;92:209-20.
- 145. Rivas-Santiago B, Hernandez-Pando R, Carranza C, et al. Expression of cathelicidin LL-37 during *Mycobacterium tuberculosis* infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. Infection and Immunity 2008;76:935-41.
- 146. Ta A, Thakur BK, Dutta P, et al. Double-stranded RNA induces cathelicidin expression in the intestinal epithelial cells through phosphatidylinositol 3-kinase-protein kinase Czeta-Sp1 pathway and ameliorates shigellosis in mice. Cell Signal 2017;35:140-153.
- 147. Fernando EH, Dicay M, Stahl M, et al. A simple, cost-effective method for generating murine colonic 3D enteroids and 2D monolayers for studies of primary epithelial cell function. Americanl Journal of Physiology-Gastrointestinal and Liver 2017;313:G467-G475.
- Weischenfeldt J, Porse B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protocols 2008;2008:pdb prot5080.
- 149. Starr T, Bauler TJ, Malik-Kale P, et al. The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with *Salmonella typhimurium*. PLoS One 2018;13:e0193601.
- Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 2009;55:611-22.
- 151. Jacobsen AV, Yemaneab BT, Jass J, et al. Reference gene selection for qPCR is dependent on cell type rather than treatment in colonic and vaginal human epithelial cell lines. PLoS One 2014;9:e115592.

- 152. Nizet V, Ohtake T, Lauth X, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 2001;414:454-7.
- 153. Lopez-Saucedo C, Bernal-Reynaga R, Zayas-Jahuey J, et al. CD40 ligand deficient C57BL/6 mouse is a potential surrogate model of human X-linked hyper IgM (X-HIGM) syndrome for characterizing immune responses against pathogens. Biomedical Research International

= 2015; 2015: 679850.

- 154. Manko A, Motta JP, Cotton JA, et al. Giardia co-infection promotes the secretion of antimicrobial peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. PLoS One 2017;12:e0178647.
- 155. Kishida K, Kohyama M, Kurashima Y, et al. Negative regulation of DSS-induced experimental colitis by PILRalpha. International Immunology 2015;27:307-14.
- Dann SM, Eckmann L. Innate immune defenses in the intestinal tract. Current Opinion in Gastroenterology 2007;23:115-20.
- 157. Sagaidak S, Taibi A, Wen B, et al. Development of a real-time PCR assay for quantification of *Citrobacter rodentium*. Journal of Microbiological Methods 2016;126:76-7.
- 158. Crepin VF, Collins JW, Habibzay M, et al. *Citrobacter rodentium* mouse model of bacterial infection. Nature Protocols 2016;11:1851-76.
- 159. Gewirtz AT, Rao AS, Simon PO, Jr., et al. Salmonella typhimurium induces epithelial IL-8 expression via Ca(2+)-mediated activation of the NF-kappa B pathway. The Journal of Clinical Investigation 2000;105:79-92.
- Huang LC, Petkova TD, Reins RY, et al. Multifunctional roles of human cathelicidin (LL-37) at the ocular surface. Investigative Ophthalmology & Visual Science 2006;47:2369-80.

- 161. Zhang J, Jiao Y, Hou S, et al. S100A4 contributes to colitis development by increasing the adherence of *Citrobacter rodentium* in intestinal epithelial cells. Scientific Reports 2017;7:12099.
- 162. Mookherjee N, Brown KL, Bowdish DM, et al. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. Journal of Immunology 2006;176:2455-64.
- Marin M, Holani R, Shah CB, et al. Cathelicidin modulates synthesis of Toll-like receptors (TLRs) 4 and 9 in colonic epithelium. Molecular Immunology 2017;91:249-258.
- 164. Marin M, Holani R, Blyth GAD, et al. Human cathelicidin improves colonic epithelial defenses against *Salmonella* Typhimurium by modulating bacterial invasion, TLR4 and pro-inflammatory cytokines. Cell Tissue and Research 2019;376:433-442.
- 165. Hornef MW, Frisan T, Vandewalle A, et al. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. The Journal of Experimental Medicine 2002;195:559-70.
- Seil M, Nagant C, Dehaye JP, et al. Spotlight on hguman LL-37, an immunomodulatory peptide with promising cell-penetrating properties Pharmaceuticals (Basel) 2010;3:3435-3460.
- 167. Hollenberg MD, Fishman PH, Bennett V, et al. Cholera toxin and cell growth: role of membrane gangliosides. Proceedings of the National Academy of Sciences of the United States of America 1974;71:4224-8.
- 168. Bennett V, Craig S, Hollenberg MD, et al. Structure and function of cholera toxin and hormone receptors. Journal of Supramolecular Structure 1976;4:99-120.
- Mukaida N, Okamoto S, Ishikawa Y, et al. Molecular mechanism of interleukin-8 gene expression. Journal of Leukocyte Biology 1994;56:554-8.

- 170. Wiehler S, Proud D. Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection. American Journal of Physiology- Lung and Molecular Physiology 2007;293:L505-L515.
- 171. Toumi F, Neunlist M, Denis MG, et al. Vasoactive intestinal peptide induces IL-8 production in human colonic epithelial cells via MAP kinase-dependent and PKA-independent pathways. Biochemical and Biophysical Research Communications 2004;317:187-91.
- 172. De S, Zhou H, DeSantis D, et al. Erlotinib protects against LPS-induced endotoxicity because TLR4 needs EGFR to signal. Proceedings of the National Academy of Sciences of the United States of America 2015;112:9680-5.
- Verjans ET, Zels S, Luyten W, et al. Molecular mechanisms of LL-37-induced receptor activation: An overview. Peptides 2016;85:16-26.
- Wu D, LaRosa GJ, Simon MI. G protein-coupled signal transduction pathways for interleukin-8. Science 1993;261:101-3.
- 175. Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. Journal of Immunology 2006;176:3044-52.
- 176. Bei Y, Pan LL, Zhou Q, et al. Cathelicidin-related antimicrobial peptide protects against myocardial ischemia/reperfusion injury. BMC Medicine 2019;17:42.
- 177. Coorens M, Schneider VAF, de Groot AM, et al. Cathelicidins inhibit *Escherichia coli*induced TLR2 and TLR4 activation in a viability-dependent manner. Journal of Immunology 2017;199:1418-1428.
- Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. The Journal of Biological Chemistry 2002;277:7059-65.

- 179. Kuroda K, Okumura K, Isogai H, et al. The human cathelicidin antimicrobial peptide LL-37 and mimics are potential anticancer drugs. Frontiers in Oncology 2015;5:144.
- 180. Sugi Y, Takahashi K, Kurihara K, et al. Post-transcriptional regulation of tollinteracting protein in the intestinal epithelium. PLoS One 2016;11:e0164858.
- Li T, Hu J, Li L. Characterization of Tollip protein upon lipopolysaccharide challenge. Molecular Immunology 2004;41:85-92.
- 182. Sakoulas G, Kumaraswamy M, Kousha A, et al. Interaction of Antibiotics with Innate Host Defense Factors against Salmonella enterica Serotype Newport. mSphere 2017;2.
- 183. Iimura M, Gallo RL, Hase K, et al. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. Journal of Immunology 2005;174:4901-7.
- 184. Mann EA, Harmel-Laws E, Cohen MB, et al. Guanylate cyclase C limits systemic dissemination of a murine enteric pathogen. BMC Gastroenterology 2013;13:135.
- 185. Eckmann L, Jung HC, Schurer-Maly C, et al. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin-8. Gastroenterology 1993;105:1689-97.
- 186. Pinheiro da Silva F, Gallo RL, Nizet V. Differing effects of exogenous or endogenous cathelicidin on macrophage toll-like receptor signaling. Immunology and Cell Biology 2009;87:496-500.
- 187. Hemshekhar M, Choi KG, Mookherjee N. Host defense peptide LL-37-mediated chemoattractant properties, but not anti-inflammatory cytokine IL-1RA production, is selectively controlled by Cdc42 Rho GTPase via G protein-coupled receptors and JNK mitogen-activated protein kinase. Frontiers in Immunology 2018;9:1871.

- 188. Bociek K, Ferluga S, Mardirossian M, et al. Lipopolysaccharide phosphorylation by the WaaY kinase affects the susceptibility of *Escherichia coli* to the human antimicrobial peptide LL-37. The Journal of Biological Chemistry 2015;290:19933-41.
- 189. Wang G. Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. J. Biol. Chem. 2008;283:32637-43.
- 190. Yamasaki K, Di Nardo A, Bardan A, et al. Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. Nature Medicine 2007;13:975-80.
- 191. Hornef MW, Normark BH, Vandewalle A, et al. Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. The Journal of Experimental Medicine 2003;198:1225-35.
- 192. Meng D, Zhu W, Shi HN, et al. Toll-like receptor-4 in human and mouse colonic epithelium is developmentally regulated: a possible role in necrotizing enterocolitis. Pediatric Research 2015;77:416-24.
- 193. Abreu MT, Arnold ET, Thomas LS, et al. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. The Journal of Biological Chemistry 2002;277:20431-7.
- 194. Grondin V, Seksik P, Dumont S, et al. Regulation of colon cancer cell proliferation and migration by MD-2 activity. Innate Immunology 2011;17:414-22.
- Bocker U, Yezerskyy O, Feick P, et al. Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll-like receptor 2 or CD14 expression. International Journal of Colorectal Disease 2003;18:25-32.
- 196. Filewod NC, Pistolic J, Hancock RE. Low concentrations of LL-37 alter IL-8 production by keratinocytes and bronchial epithelial cells in response to proinflammatory stimuli. Federation of European Microbiological Societies Immunology and Medical Microbiology 2009;56:233-40.

- 197. Lai Y, Adhikarakunnathu S, Bhardwaj K, et al. LL37 and cationic peptides enhance TLR3 signaling by viral double-stranded RNAs. PLoS One 2011;6:e26632.
- 198. Jijon HB, Panenka WJ, Madsen KL, et al. MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a post-transcriptional mechanism. American Journal of Cellular Physiology 2002;283:C31-41.
- 199. Dean JL, Sarsfield SJ, Tsounakou E, et al. p38 Mitogen-activated protein kinase stabilizes mRNAs that contain cyclooxygenase-2 and tumor necrosis factor AU-rich elements by inhibiting deadenylation. The Journal of Biological Chemistry 2003;278:39470-6.
- 200. Ang Z, Koean RAG, Er JZ, et al. Novel AU-rich proximal UTR sequences (APS) enhance CXCL8 synthesis upon the induction of rpS6 phosphorylation. PLoS Genetics 2019;15:e1008077.
- 201. Negroni A, Cucchiara S, Stronati L. Apoptosis, Necrosis, and Necroptosis in the Gut and Intestinal Homeostasis. Mediators Inflamm 2015;2015:250762.
- 202. Lebeis SL, Bommarius B, Parkos CA, et al. TLR signaling mediated by MyD88 is required for a protective innate immune response by neutrophils to *Citrobacter rodentium*. Journal of Immunology 2007;179:566-77.
- 203. Wine E, Shen-Tu G, Gareau MG, et al. Osteopontin mediates *Citrobacter rodentium*induced colonic epithelial cell hyperplasia and attaching-effacing lesions. American Journal of Pathology 2010;177:1320-32.
- 204. Williams JM, Duckworth CA, Watson AJ, et al. A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. Disease Models & Mechanisms 2013;6:1388-99.
- 205. Simmons CP, Goncalves NS, Ghaem-Maghami M, et al. Impaired resistance and enhanced pathology during infection with a noninvasive, attaching-effacing enteric

bacterial pathogen, *Citrobacter rodentium*, in mice lacking IL-12 or IFN-gamma. Journal of Immunology 2002;168:1804-12.

- 206. Baker J, Brown K, Rajendiran E, et al. Medicinal lavender modulates the enteric microbiota to protect against *Citrobacter rodentium*-induced colitis. Americal Journal of Physiology: Gastrointeststinal and Liver Physiology 2012;303:G825-36.
- 207. Li F, Wang HD, Lu DX, et al. Neutral sulfate berberine modulates cytokine secretion and increases survival in endotoxemic mice. Acta Pharmacologica Sinica 2006;27:1199-205.
- 208. Mader JS, Marcet-Palacios M, Hancock RE, et al. The human cathelicidin, LL-37, induces granzyme-mediated apoptosis in cytotoxic T lymphocytes. Experimental Cell Research 2011;317:531-8.
- 209. Maillard MH, Bega H, Uhlig HH, et al. Toll-interacting protein modulates colitis susceptibility in mice. Inflammatory Bowel Diseases 2014;20:660-70.
- 210. Wan N, Liu X, Zhang XJ, et al. Toll-interacting protein contributes to mortality following myocardial infarction through promoting inflammation and apoptosis. British Journal of Pharmacology 2015;172:3383-96.
- 211. Li M, Feng B, Wang L, et al. Tollip is a critical mediator of cerebral ischaemiareperfusion injury. Journal of Pathology 2015;237:249-62.
- Singer AJ, Clark RA. Cutaneous wound healing. The New England Journal of Medicine 1999;341:738-46.
- 213. Hou Y, Lu X, Zhang Y. IRAK inhibitor protects the intestinal tract of necrotizing enterocolitis by inhibiting the Toll-like receptor (TLR) inflammatory signaling pathway in rats. Medical Science Monitor 2018;24:3366-3373.

- 214. Kumazoe M, Yamashita M, Nakamura Y, et al. Green tea polyphenol EGCG upregulates Tollip expression by suppressing elf-1 expression. Journal of Immunology 2017;199:3261-3269.
- 215. Chen Y, Choong LY, Lin Q, et al. Differential expression of novel tyrosine kinase substrates during breast cancer development. Molecular & Cellular Proteomics 2007;6:2072-87.
- 216. Liu NS, Loo LS, Loh E, et al. Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor. The European Molecular Biology Journal 2009;28:3485-99.
- 217. Katoh Y, Imakagura H, Futatsumori M, et al. Recruitment of clathrin onto endosomes by the Tom1-Tollip complex. Biochemical and Biophysical Research Communications 2006;341:143-9.
- 218. Hayashi M, Kuroda K, Ihara K, et al. Suppressive effect of an analog of the antimicrobial peptide of LL37 on colon cancer cells via exosome-encapsulated miRNAs. International Journal of Molecular Medicine 2018;42:3009-3016.
- 219. Larsen MT, Hager M, Glenthoj A, et al. miRNA-130a regulates C/EBP-epsilon expression during granulopoiesis. Blood 2014;123:1079-89.
- 220. Wang J, Cheng M, Law IKM, et al. Cathelicidin suppresses colon cancer metastasis via a P2RX7-dependent mechanism. Molecular Therapy Oncolytics 2019;12:195-203.
- 221. Zhu H, Dougherty U, Robinson V, et al. EGFR signals downregulate tumor suppressors miR-143 and miR-145 in Western diet-promoted murine colon cancer: role of G1 regulators. Molecular Cancer Research 2011;9:960-75.
- 222. Seike M, Goto A, Okano T, et al. MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers. Proceedings of the National Academy of Sciences of the United States of America 2009;106:12085-90.

- 223. Diederichs S, Jung S, Rothenberg SM, et al. Coexpression of Argonaute-2 enhances RNA interference toward perfect match binding sites. Proceedings of the National Academy of Sciences of the United States of America 2008;105:9284-9.
- 224. Chendrimada TP, Gregory RI, Kumaraswamy E, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 2005;436:740-4.
- 225. Shen J, Xia W, Khotskaya YB, et al. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature 2013;497:383-7.
- 226. Adams BD, Claffey KP, White BA. Argonaute-2 expression is regulated by epidermal growth factor receptor and mitogen-activated protein kinase signaling and correlates with a transformed phenotype in breast cancer cells. Endocrinology 2009;150:14-23.
- 227. Dittmann K, Mayer C, Czemmel S, et al. New roles for nuclear EGFR in regulating the stability and translation of mRNAs associated with VEGF signaling. PLoS One 2017;12:e0189087.
- 228. Hagar JA, Powell DA, Aachoui Y, et al. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science 2013;341:1250-3.
- 229. Xu C, Ghali S, Wang J, et al. CSA13 inhibits colitis-associated intestinal fibrosis via a formyl peptide receptor like-1 mediated HMG-CoA reductase pathway. Scientific Reports 2017;7:16351.
- 230. Su Y, Wang H, Mishra B, et al. Nanofiber dressings topically delivering molecularly engineered human cathelicidin peptides for the treatment of biofilms in chronic wounds. Molecular Pharmaceutics 2019;16:2011-2020.
- 231. Wong CC, Zhang L, Wu WK, et al. Cathelicidin-encoding *Lactococcus lactis* promotes mucosal repair in murine experimental colitis. Journal of Gastroenterology and Hepatology 2017;32:609-619.

## **APPENDIX- COPYRIGHT PERMISSIONS**

**A. Copyright approval for article-** Holani R, Shah C, Haji Q, Inglis GD, Uwiera RR, Cobo ER (2016). Proline-arginine rich (PR-39) cathelicidin: Structure, expression and functional implication in intestinal health. *Comparative Immunology Microbiology Infectious Diseases*, 49: 95-101.

From: Permissions Helpdesk permissionshelpdesk@elsevier.com

**Sent:** Friday, January 3, 2020 6:12:16 AM

To: Ravi Holani <<u>ravi.holani@ucalgary.ca</u>>

Subject: Re: Permission to include article into thesis [200103-001498]

Dear Ravi Holani,

Thank you for your query.

Please note that, as one of the authors of this article, you retain the right to reuse it in your thesis/dissertation. You do not require formal permission to do so. You are permitted to post this Elsevier article online if it is embedded within your thesis. You are also permitted to post your Author Accepted Manuscript online.

However posting of the final published article is prohibited.

## B. Copyright approval for article- Holani R, Marin M, Kastelic J, Cobo ER (2018).

Antimicrobial peptides in gastrointestinal disease. Chapter "Host defense peptides as innate immunomodulators in the pathogenesis of colitis". Editor: Dr. Chi Hin Cho (Southwest Medical University, Luzhou, China). Elsevier B978-0-12-814319.

From: <u>no-reply@copyright.com</u> <<u>no-reply@copyright.com</u>>

Sent: Thursday, January 16, 2020 11:48:39 PM

To: Ravi Holani <<u>ravi.holani@ucalgary.ca</u>>

Subject: RESPONSE REQUIRED for your request to Elsevier

Dear Mr. Ravi Holani,

Elsevier has approved your recent request described below. Before you can use this content, **you must accept** the license fee and terms set by the publisher.

Use this <u>link</u> to accept (or decline) the publisher's fee and terms for this order.

# **Order Summary**

Licensee:	University of Calgary
Order Date:	Jan 3, 2020
Order Number:	501537537
Publication:	Elsevier Books
Title:	Antimicrobial Peptides in Gastrointestinal Diseases
Type of Use:	reuse in a thesis/dissertation
Order Total:	0.00 CAD