Citrobacter rodentium and *Escherichia coli* interactions with mucus producing epithelial cells

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ABSTRACT

The epithelial cells together with the mucus layer protect the host from noxious luminal substances and pathogenic invasion. Pathogens have evolved numerous strategies to circumvent these barriers and mount infection. *C. rodentium* is a murine model for the attaching and effacing intestinal *E. coli* (EPEC, EHEC) and share similar virulence strategies to infect their host.

We found that the colonic mucus layer is dynamically regulated by *C. rodentium* and the ensuing cytokine response. The cytokine profile investigated during the course of infection indicated a shift from pro- to an anti-inflammatory type of response at times of increased mucus layer thickness. The *in vitro* effect of signature cytokines of pro- and anti-inflammatory responses and the pathogens (EPEC, ETEC and *C. rodentium*) indicated that changes in mucin production and secretion are affected by the combined impact of these factors. The anti-inflammatory cytokine IL-4 alleviated mitochondrial dysfunction *in vitro* and accelerated mucin production and secretion, especially in the presence of EPEC, ETEC and *C. rodentium*. *In vivo* IL-4 treatment improved mitochondria and barrier functions and colitis symptoms. Similarly, VIP alleviated mitochondrial dysfunction during infection. The lack of Fpr2 lead to decreased barrier function and increased susceptibility to *C. rodentium* and EPEC infection. Harnessing the host's response to pathogens could improve the intestinal mucus barrier function by enhancing mucosal healing and shortening the duration of infection.

Keywords: A/E pathogens, cytokines, epithelium, mitochondria, mucus

SAMMANFATTNING PÅ SVENSKA

Escherichia coli (*E. coli*) är bakterier som lever i tarmkanalen hos både människor och djur och är en del av den normala tarmfloran. Dock existerar sjukdomsalstrande stammar så kallade patogener som kan orsaka utbrott av diarré och tarminflammation med dödligt utfall. Infektionen drabbar främst barn och är vanligast förekommande i utvecklingsländer. *Citrobacter rodentium* (*C. rodentium*) ger liknande symptom hos möss som patogena typer av *E. coli* (EPEC/EHEC) och används främst i musmodeller för att studera värdens försvar och bakteriens virulens. Ett finmaskigt slemlager (mukus) beklär slemhinnans ytceller (epitelceller) och bildar en skyddande barriär mellan tarminnehållet och epitelcellerna. Mukusproduktionen är reglerad av bakterieprodukter samt små proteiner och signalmolekyler (cytokiner) som främst produceras av immunförsvaret. För att kunna orsaka sjukdom måste patogenerna först ta sig igenom mukusbarriären innan de kan få fäste på epitelcellytan. Därefter injicerar bakterierna effektormolekyler genom epitelcellväggarna via ett sekretionssytem som påminner om en injektionsspruta. Effektormolekylerna aktiverar tarmens immunsystem som svarar med att utsöndra cytokiner och andra antibakteriella peptider som i sin tur leder till inflammation. Vissa av effektormolekylerna är inriktade mot mitokondrierna i cellen. Mitokondrier är en typ av organeller som förser eukaryota celler med energi i form av adenosintrifosfat. De fyller ytterligare väsentliga funktioner som celldelning, cellsignalering och pH-reglering. Rubbningar i mitokondriernas funktion t.ex. vid infektion kan leda till programmerad celldöd (apoptos). Vid apoptos frigörs cellinnehåll, som bland annat formylpeptider vilket bakterier också uttrycker. De binder till formylpeptidreceptor 2 (Fpr2) som befinner sig på immuncellytorna och vägleder cellerna till vävnaden. Tidigare har man visat att Fpr2 uttrycks på epitelceller och spelar en avgörande roll i läkningsprocessen av skadade epitelceller. Tidigare studier har visat att möss infekterade med *C. rodentium* till en början får ett tunt mukuslager som sedan under eradikering av bakterierna ökar i tjocklek. Vad gäller den dynamiska förändringen i mukuset har vi kunnat visa att cytokiner och patogener påverkar produktionstakt och omsättning av mucus både i möss och odlade celler. Vi upptäckte dessutom att tillsatsen av en av cytokinerna, Interleukin-4 (IL-4), ökar produktionstakten och omsättningen av mukus i odlade celler och skyddar dem mot andra cytokiner som är viktiga för eradikering av patogener, men

skadliga för de eukaryota cellerna. Vidare kunde vi visa att IL-4 skyddar mitokondrierna mot både patogener och skadliga cytokiner. Vasoactive intestinal peptide (VIP) har tidigare har visat sig lindra tarminflammation genom att ändra på cytokinmiljön. Vi fann en minsking av VIP i tarmvävnaden hos infekterade möss under inflammation. Resultaten för behandling med VIP tyder på att den skyddar tarmen mot en del av de infektionsrelaterade skadorna genom att skydda mitokondrierna. De infekterade mössen blev dock över lag sjukare av VIP behandlingen. Vidare har vi kunnat visa att IL-4 behandling, i infekterade möss, ökar produktionstakten och omsättningen av mukus och skapar ett tätare mukuslager som är mindre genomsläppligt. Dessutom reducerar IL-4 antalet bakterier i kontakt med epitelet och har en lindrande effekt mot inflammationen. Vi har även kunnat visa att möss som saknar Fpr2 är mer känsliga för infektion, har fler bakterier i kontakt med epitelet och högre grad av tarminflammation. De har dessutom minskad omsättning och produktion av mukus vilket troligtvis beror på den förändrade cytokinprofil i samband med infektionen.

Sammanfattningsvis kan fastställas att, cytokinmiljön påverkar produktionen och omsättningen av mukus under infektionen vilket i sin tur leder till dynamiska förändringar i mukuslagret. Genom att komplementera värdens cytokinsvar kan vi förbättra slemhinnans försvar mot tarmpatogener.

LIST OF PAPERS

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

I. Maiti AK, Sharba S, Navabi N, Forsman H, Fernandez HR, Linden SK
IL-4 protects the mitochondria against TNFα and IFNγ induced insult during clearance of infection with *Citrobacter rodentium* and *Escherichia coli*
Scientific Reports **2015**; 5: 15434.

II. Maiti AK*, Sharba S*, Navabi N, Linden SK.
Colonic levels of vasoactive intestinal peptide decrease during infection and exogenous VIP protects epithelial mitochondria against the negative effects of IFNγ and TNFα induced during *Citrobacter rodentium* infection
PLoS One **2018**; 13(9)**:**e0204567.
*Equal contribution

III. Sharba S*, Navabi N*, Padra M, Persson JA, Quintana-Hayashi MP, Gustafsson JK, Szeponik L, Venkatakrishnan V, Sjöling Å, Nilsson S, Quiding-Järbrink M, Johansson MEV, Linden SK
Interleukin 4 induces rapid mucin transport, increases mucus thickness and quality and decreases colitis and *Citrobacter rodentium* in contact with epithelial cells
Virulence **2019**; 10(1)**:** 97-117.
*Equal contribution

IV. Sharba S, Venkatakrishnan V, Padra M, Winther M, Gabl M, Sundqvist M, Wang J, Forsman H, Lindén SK
Formyl peptide receptor 2 orchestrates mucosal protection against *Citrobacter rodentium* infection
Virulence **2019**; 10(1): 610-624.

CONTENT

ABBREVIATIONS

NB: Protein names in capital letters refer to human proteins, whereas protein names in lower case refer to mouse.

AB-PAS	Alcian blue - Periodic acid-Shiff
A/E	Attaching and effacing
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
Ca^{+}	Calcium
CF	Colonization factor
CFU	Colony forming units
CpG	5'-C-phosphate-G-3'
C. rodentium	*Citrobacter rodentium*
DAPT	*N*-[(3,5-Difluorophenyl)acetyl]-l-alanyl-2-phenyl]glycine-1,1-dimethylethyl -ester
DAMP	Damage associated molecular patterns
DSS	Dextran sodium sulphate
EAEC	Enteroaggregative *Escherichia coli*
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
E. histolytica	*Entamoeba histolytica*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ER	Endoplasmic reticulum
EspF	Secreted effector protein F
EtaA	ETEC autotransporter A
ETC	Electron transport chain
ETEC	Enterotoxigenic *Escherichia coli*
$FADH_2$	Flavin adenine dinucleotide
fMet	N-formylmethionine
FPR/Fpr	Formyl peptide receptor
GalNAc	*N*-acetylgalactosamine
GalNAz	*N*-azidoacetylgalactosamine
GlcNAc	*N*-Acetylglucosamine
H. pylori	*Helicobacter pylori*
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin

IL-4R	Interleukin-4 receptor
LEE	Locus of enterocyte effacement
LnCP	Legionellanucleotide carrier protein B
LPS	Lipopolysaccharide
LT	Heat-labile
MALT	Mucosa associated lymphoid tissue
MAMP	Microbial associated molecular patterns
Map	Mitochondrial associated protein
mtDNA	Mitochondrial DNA
Muc	Mucin
MyD88	Myeloid differentiation primary response gene 88
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nlrp3	NACHT, LRR and PYD domains-containing-protein 3
PAI	Pathogenicity island
PAMP	Pathogen associated molecular patterns
PI	Post-infection
Pic	Protein involved in colonization
PRR	Pathogen recognition receptors
PTS	Proline, threonine and serine rich
ROS	Reactive oxygen species
SipB	*Salmonella* invasion protein B
SPATE	Serine protease autotransporters of the enterobacteriaceae
SPDEF	SAM pointed domain containing ETS transcription factor
ST	Heat-stable
Stat6	Signal transducer and activator of transcription 6
StcE	Secreted protease of C1 esterase inhibitor
T3SS	Type III secretion system
TAMRA	Tetramethylrhodamine
TGF-b1	Transforming growth factor-b-activated kinase 1
Th	T-helper
Tir	Translocated intimin receptor
TLR	Toll-like receptors
TNBS	2,4,6-Trinitrobenzenesulfonic acid solution
TNF-α	Tumor necrosis factor-alpha
VacA	Vacuolating cytotoxin A
VIP	Vasoactive intestinal peptide
VopE	Effector protein of *Vibrio cholera*
WT	Wild-type

1 INTRODUCTION

1.1 The intestinal tract

The intestinal tract is the largest microbial ecosystem in our body, it covers an enormous surface area and is responsible for nutrient uptake and fluid absorption. The intestinal tract consists of the small and large intestine or colon and is composed of four concentric tissue layers: serosa, muscularis externa, submucosa, and a single cell layer termed the mucosa. The mucosa or intestinal epithelium facing the lumen consists of a single layer of columnar epithelial cells made up of four differentiated cell types that absorb nutrients, and secrete mucus, hormones and antimicrobial agents that protect the host from noxious luminal substances, including enteric microorganisms [1-3]. A protective sheet of secreted mucus covering the colonic epithelium is the first defense barrier. The highly hydrated and viscous mucus gel in the intestine is mainly comprised by the MUC2 mucin, but also contains a range of other components with defensive properties, such as antimicrobial peptides, Immunoglobulin A (IgA), and complement factors [2, 4]. The mucus layer is divided into an inner firm layer nearly devoid of microbes, and an outer loose layer harboring the commensal flora [5]. The inner layer is densely packaged, stratified, impermeable to bacteria-sized beads and difficult to physically dislodge from the tissue [5-7]. Nevertheless, the outer layer is home to some 10^{14} microbes constituted by up to 1000 different species that ferment undigested food and degrade mucus as an energy source [8, 9]. In addition, removal of trapped luminal material coupled with a short mucin half-life requires constant renewal of mucus and is reflected by the fast turnover of MUC2 in surface goblet cells in the colon [5, 10].

1.2 *Escherichia coli*

Escherichia coli (*E. coli*) is a rod-shaped gram-negative facultative anaerobe belonging to the family of *Enterobacteriaceae* residing in the outer mucus layer of the colon as part of the microflora. *E. coli* can be both harmless and pathogenic. The commensals contribute to the protection against colonization

of the pathogenic strains responsible for enteric diseases. The diarrheagenic *E. coli*, based on their genetic and phenotypic traits are enteroinvasive *E coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enterohaemorrhagic *E. coli* (EHEC) [11].

1.2.1 ETEC

ETEC is a major cause of infantile and travelers' diarrhea in developing countries. Since ETEC is a non-invasive pathogen, the bacterium relies on colonization factors (CFs) that mediate adherence to the small intestinal epithelium, and secretion of enterotoxins that stimulate net intestinal fluid secretion resulting in watery diarrhea [12]. An increase in luminal ion concentration caused by the heat-labile (LT) and/or heat-stable (ST) enterotoxin stimulate chloride secretion while inhibiting sodium chloride absorption, leading to the diffusion of water from the mucosa into the lumen [11]. To reach the epithelial surface ETEC employs additional mechanisms to overcome the mucus barrier. The metalloprotease ETEC autotransporter A (EtaA) and the CF yghj are capable of degrading Muc2 and facilitate toxin delivery to the epithelium *in vitro* [13, 14].

1.2.2 A/E Pathogens

EPEC and EHEC cause acute gastroenteritis in humans [11] affecting the small and large intestine, respectively. EPEC is a pediatric disease which causes acute watery diarrhea mainly in developing countries, while EHEC is a zoonotic pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome affecting primarily children and the elderly in developed countries [11, 15, 16]. Although these two microorganisms cause different diseases, the hallmark of infection is attaching and effacing (A/E) histopathological lesions characterized by intimate attachment to host epithelial cells, effacement of microvilli and cytoskeletal rearrangement [11, 17]. The locus of enterocyte effacement (LEE) located on a pathogenicity island (PAI) encodes the outer membrane protein intimin used for adhesion and its translocated intimin receptor (Tir) is inserted into the host cell membrane for intimate attachment to epithelial cells [17]. Moreover, LEE contains genes for a type III secretion system (T3SS) that functions as a molecular syringe for the transfer of over twenty effector proteins [18-20]. Pathogenic strains of *E. coli* do not naturally

colonize the mouse intestine and require pre-treatment with antibiotics [21] or diet alterations [22] which would modify the local gut environment. Instead, as EPEC and EHEC are poorly pathogenic in rodent, the mouse pathogen *Citrobacter rodentium* (*C. rodentium*) is used as a model to elucidate the A/E pathogenesis of infection [17, 23, 24]. *C. rodentium* carries a homologue of the LEE PAI and uses similar virulence strategies as described above for EPEC and EHEC to infect mice [25]. Oral challenge with $>10^8$ colony forming units (CFU) initially results in colonization of the caecum, leading to heavy colonization of the distal colon within a few days [26]. *C. rodentium* causes transmissible murine colonic hyperplasia resulting in epithelial cell hyperproliferation in the distal colon and thickening of the colonic tissue [27]. The infection is self-limiting and most mouse strains survive, clearing *C. rodentium* within three to four weeks post-infection [26, 28].

1.3 Mitochondria

The mitochondria supply eukaryotic cells with energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation. This mechanism involves passing of electrons donated by NADH and $FADH_2$, through the electron transport chain (ETC) consisting of a set of membrane proteins referred to as complex I-IV. The flow of electrons between complexes and movement of protons across the matrix creates a proton gradient. ATP is produced from adenosine diphosphate (ADP) and phosphate as protons re-enter the matrix via complex V or ATP synthase [29]. However, the mitochondrion is not merely a powerhouse for cells, but a multifunctional organelle central to the regulation of other cellular processes, including intracellular Ca^+ homeostasis, lipid metabolism, immunity and programed cell death (apoptosis) [30-32]. The endosymbiotic theory of mitochondria suggests that they have originated from energy-producing bacteria [33]. Nevertheless, mitochondria carry a separate genome of mitochondrial DNA (mtDNA) and similar to bacteria, the mtDNA is double stranded, circular, and contain CpG DNA that encode formyl peptides [29]. A subset of components of the ETC are encoded by mtDNA and translation of those components takes place inside the mitochondria [34].

1.3.1 Mitochondria and apoptosis

Apoptosis during homeostasis promotes the release of various molecules that, in the absence of pathogen associated molecular patterns (PAMPs), suppress and promote pro-inflammatory and anti-inflammatory cytokine responses, respectively [35]. Necrosis, on the other hand, occurs following injury or infection (non-physiological damage), is not programmed and may therefore trigger overt inflammation. Given their prokaryotic origin, mitochondria-derived danger associated molecular patterns (DAMPs) such as mtDNA and formylated peptides resemble bacterial PAMPs, they bind to the same pathogen recognition receptors (PRRs) on leukocytes and trigger inflammation [36] (see section 1.4.1 Fpr2). In addition, dissipation of mitochondrial membrane potential due to an increase in mitochondrial membrane permeability caused by damage to the cell results in loss of ATP, release of cytochrome c and excessive production of reactive oxygen species (ROS) [37]. The surplus of ROS production activates the inflammasome Nlrp3 which in turn activates pro-inflammatory cytokines interleukin (IL)-1β and IL-18 though caspase-1 leading to cell death [38-40]. The release of cytochrome c from the ETC to the cytoplasm activates caspase-9 that cleaves caspase-3 resulting in cell death [41].

1.3.2 Mitochondria and infection

A/E pathogens inject secreted effector protein F (EspF) that translocate into host cell mitochondria interfering with functions related to membrane potential and the release of cytochrome c [42]. Moreover, mitochondrial associated protein (Map), another effector protein injected by A/E pathogens, has been shown to alter mitochondrial membrane potential *in vitro* [43] and co-localized with mitochondria in colonic epithelial cells of *C. rodentium* infected mice [44]. Mitochondria seem to be an attractive target for pathogens with various infection strategies and delivery mechanisms of effector proteins such as the extracellular pathogens *Vibrio cholera* (VopE) [45] and *Helicobacter pylori* (VacA) [46], and the intracellular pathogens *Salmonella* Typhimurium (SipB) [47] and *Legionella pneumophila* (LnCP) [48]. Pro-inflammatory cytokines may also contribute to mitochondrial dysfunction as shown in non-intestinal tissues by inducing ROS production [49], interfering with the ETC [50], and causing cell death [51].

1.4 Pathogen recognition

Neutrophils are amongst the first immune cells to arrive at the site of injury or infection and are recruited by various chemoattractants [52]. Many of the chemoattractant receptors on neutrophils belong to the G-protein superfamily [53]. The regulation of cellular processes in response to pathogen invasion occurs mainly through receptor activation of immune cells. PRRs recognize structures conserved among microbial species known as PAMPs. DAMPs are endogenous molecules released from damaged cells and are also sensed by PRRs. Toll-like receptors (TLRs) are a class of PRRs and their activation initiate inflammatory responses by secreting cytokines, chemokines, antimicrobial proteins, and other molecules to orchestrate the tissue response against pathogens [54]. Intestinal epithelial cells (IECs) are also equipped with various innate immune receptors to detect the intrusion of foreign microorganisms. TLRs on IECs regulate epithelial homeostasis and aid in the induction of inflammatory responses against bacterial products. The deletion of molecules acting downstream of TLRs such as myeloid differentiation primary response gene 88 (MyD88), transforming growth factor-b-activated kinase 1 (TGF-b1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in mice lead to the development of spontaneous intestinal inflammation [55-57]. The expression of defensins and antimicrobial factors by IECs mainly depends on TLR/Myd88 signaling [58, 59]. TLR4 on IECs induce pro-inflammatory responses to microbes and reduce goblet cell density in the intestine [60]. In addition, mice lacking TLR2, 4, and 9 in IECs were more susceptible to dextran sodium sulphate (DSS)-induced colitis [61]. Inhibiting NF-κB in IECs impaired mucosal homeostasis and mucosal immune responses [56, 62]. Mice with IEC-specific deletion of p38α (a member of the mitogen-activated protein kinase family) show impaired recruitment of inflammatory cells to the epithelium during *C. rodentium* infection.

1.4.1 Formyl peptide receptor 2 (Fpr2)

In eukaryotic cells, protein synthesis is initiated by the amino acid methionine while, in bacteria and mitochondria, a modified methionine namely, N-formylmethionine (fMet) serves as the starting amino acid [29]. Upon pathogenic invasion or host cell injury, the formylated peptides bind to formyl peptide receptors (Fprs) which, triggers immune cell activation and

chemotaxis. However, Fprs are not restricted to cells of myeloid origin as they are found in various tissue types, including epithelial cells [63]. Fpr2 has been shown to offer protection against chemically induced colitis in mice [64], respond to various cytokine treatments [65] and accelerate healing in *in vitro* cultured epithelial cells of colonic origin [66].

1.5 Mucin synthesis

MUC2 is the main structural component of mucus in the colon and is responsible for its gel-like properties. It is a high-molecular-weight glycoprotein with heavily glycosylated PTS or mucin domains. Starting at the endoplasmic reticulum (ER), the mucin backbone is synthesized and dimerizes via the C-terminal end. The dimers are then translocated to the Golgi complex where extensive *O*-glycosylation occurs at the PTS domains followed by N-terminal oligomerization of the dimers [67-69]. Once fully glycosylated, MUC2 is tightly packaged and stored in a highly condensed and dehydrated state in goblet cell granules.

1.5.1 Breaching the mucus barrier

A prerequisite for mucosal pathogens to mount successful infection is to overcome the mucus barrier to reach the underlying epithelium. Pathogens employ different strategies to circumvent the protective mucus barrier. *H. pylori* alters the rheology of stomach mucus and use flagella to propel themselves towards the epithelium [70]. However, other bacteria and parasites use proteases to degrade mucins and alter the macromolecular structure of mucus facilitating invasion of the epithelium [2]. The metalloprotease StcE secreted by EHEC, Pic secreted by *C. rodentium,* and EtaA secreted by ETEC, belong to the serine protease autotransporters of the *enterobacteriaceae* (SPATE) protein family, display mucinase activity and likely play a role in breaking down the mucus barrier [13, 71, 72]. Mice deficient in Muc2 show increased susceptibility to *C. rodentium* infection with rapid colonization and greater pathogen burdens compared to their WT counterpart highlighting the importance of overcoming the mucus barrier to establish infection [73]. Furthermore, clearance of *C. rodentium* is delayed in mice with defective mucus exocytosis [74] and the binding of *C. rodetnium* to Muc2 [75]

may aid in the transportation of pathogens towards the lumen away from the epithelial surface.

1.5.2 Mucin production, turnover and secretion

Regulation of secretion is either constitutive, with low-level secretion, or accelerated in response to external stimuli [76]. Following stimulation, vesicles fuse with the cell membrane and secrete the densely packaged MUC2 oligomers [77]. Upon release the packed mucin oligomers undergo hydration and massive volume expansion stacking the unfolded netlike sheets in the luminal direction, thus maintaining a dense striated inner mucus layer [78]. Mucus is lost to peristalsis and degradation by luminal bacteria, and the thickness of the mucus layer is usually maintained by constitutive production and secretion of mucins coupled to the conversion rate of the inner- to the outer mucus layer. In accelerated secretion or compound exocytosis, the majority of the goblet cell vesicles are almost completely emptied in response to stimuli [77]. Microbial invasion of the mucosa caused by a defect in the mucus layer [5] or pathogenic invasion [2] could lead to alterations related to mucin structure and synthesis and may affect the rate at which mucus is secreted [79]. Glycoprotein labeling with the azide-modified *N*-acetylgalactosamine (GalNAz) allows tracking of mucin production and secretion after being incorporated into the core region of mucin *O*-glycans during biosynthesis [10] (**Figure 1 B1 and B2**). The rate at which metabolically labeled mucin is being secreted into the lumen in non-infected mice varies depending on the location being studied, 10 - 12 h in the stomach [79], and 6 - 8 h in the small and large intestine [10, 80].

Some pathogens affect mucus production and/or secretion or mucin biosynthesis. The enteric protozoan parasite *Entamoeba histolytica* (*E. histolytica*) depletes mucin storage in gerbils prior to invasion of colonic epithelial cells [81] *E. histolytica* also increase mucus secretion in *in vitro* cultured colonic epithelial cells [82] and in the rat colon [83]. *Nippostrongylus brasiliensis* increase small intestinal mucin gene expression and induce changes in the glycosylation pattern of mucins during expulsion of the parasite from the rat small intestine [84]. During acute *Trichurius muris* infection IL-13 alters mucin glycosylation shifting from sulphated to sialylated mucins for proper expulsion of the worm [85]. Inhibition of mucin secretion caused by the gastric pathogen *H. pylori* alter human stomach

mucins by decreasing mucin synthesis [86], reducing exocytosis *in vitro* [87], and decreasing mucin turnover and secretion in mice [79] possibly to create a more stable niche for itself.

1.6 Mucosal immunity and cytokines

The GI tract is the largest immunological organ in the body exposed to the external environment. The mucosa associated lymphoid tissue (MALT) consists of immune cells and lymphoid structures. The innate immune response to infection is activated by PRRs that bind PAMPs. This results in secretion of antimicrobial peptides, cytotoxic molecules, phagocytosis, and complement activation. IECs and immune cells are involved in the innate host defense against invading pathogens. In addition to mucins, IECs secrete antimicrobial peptides [88] and cytokines, primarily IL-8 [89]. However, T-helper (Th) cells secrete cytokines that elicit cellular or humoral immune responses. T helper cells can be subdivided into Th1, Th2 and T17 cells based on their cytokine profile. Th1 and Th17 cells are involved in cellular immune responses and secrete the signature cytokines interferon-gamma (IFN-γ)/ tumor necrosis factor-alpha (TNF-α) and IL-17/IL-22, respectively [90]. IFN-γ and TNF-α have been shown to contribute to host defense and enhance the innate immune response against *C. rodentium* [91-93]. Moreover, IL-22 plays a crucial role in the protection against *C. rodentium* [94], and IL-17 aids in clearance but is also responsible for the ensuing tissue pathology [95]. Th2 cells favor a humoral response; they secrete their signature cytokines IL-4 and IL-13 and engage in the host response against parasites [90]. Additionally, Th2 cytokines regulate B cell function by stimulating antibody production such as IgG, crucial for clearance of *C. rodetium* [96]. These cytokines induce goblet cell metaplasia [97, 98] and up-regulate gel-forming mucin gene expression through signal transducer and activator of transcription 6 (Stat6) activation via IL-4 receptor (IL-4R) in the murine airways [99]. On the same note, *in vitro* stimulation of intestinal cell lines by IL-4 and IL-13 activated Stat6 which in turn upregulated the transcription of trefoil factor 3, a peptide and goblet cell product that repairs and restores mucosal homeostasis [100].

1.7 Vasoactive intestinal peptide (VIP)

Neuropeptides, in addition to microbial components, cytokines, nitric oxide [101], and cholinergic stimulants [77], can induce accelerated mucus secretion [102]. Neurons innervating the intestinal epithelium regulate intestinal physiology and function in many aspects through secretion of a vast number of neurotransmitters including the vasoactive intestinal peptide (VIP) [103, 104]. VIP inhibits pro-inflammatory immune responses [105] and its anti-inflammatory properties have been found in various tissue types including those of the gastrointestinal tract [106]. A loss of VIP-neurons have been associated with inflammatory bowel disease [107]. Moreover, it has been shown that mice lacking VIP ($VIP^{-/-}$) are more susceptible to chemically induced colitis with increased apoptosis and disruption of mucosal homeostasis [108]. *C. rodentium*-induced epithelial damage was limited by prophylactic treatment with VIP observed at mid-point of infection [109]. Furthermore, a decrease in symptoms associated with chemically-induced colitis was observed in mice treated with VIP following disease onset [110]. Conversely, in other studies, $VIP^{-/-}$ mice seem to be protected against symptoms related to chemically-induced colitis, and in WT mice the inhibition of VIP resulted in a milder phenotype [111]. However, others have shown that VIP protects mitochondrial function in lung epithelial cells both *in vivo* [112] and *in vitro* [113].

2 AIM

General aim

The aim of this thesis was to study the role of epithelial cell health and mucin production and secretion in relation to A/E pathogens and the host immune response to infection.

Specific aims:

- To investigate the effect of *C. rodentium* and the cytokine environment ensuing from infection on mucin production and secretion
- To improve the host response to pathogens during infection by altering mucus quality and increasing mucin turnover and secretion in the colon
- To examine mitochondrial function during *C. rodentium* infection and differential cytokine expression
- To investigate the role of Fpr2 during *C. rodentium* infection

3 METHODOLOGY

The methods used in this thesis are included in Papers (I-IV). Here, we discuss the *in vitro* mucosal surfaces, metabolic labelling used and special considerations taken into account with relation to this method.

3.1.1 *In vitro* mucosal surfaces

The use of *in vitro* models to understand the mechanisms of infection or host-pathogen interactions on barrier function allows for easy pharmacological intervention. The *in vitro* mucosal surfaces referred to in this thesis (Paper I-IV) are based on a three-dimensional culture of HT29-MTX-E12 cells (derived from MTX selected colorectal adenocarcinoma cells). The method is discussed in detail in Quintana-Hayashi and Lindén 2018 [114], but in brief cells seeded on Snapwells are allowed to grow confluent for 6 days followed by chemical stimulation with the Notch γ-secretase inhibitor (DAPT) for another 6 days under air-liquid interphase and lastly mechanical stimulation under semi-wet interphase for 22 days. At day 34 post-seeding, these cells have formed an *in vivo*-like mucosal surface with polarized epithelial cells, functional tight junctions, apical mucus secretion, and a mucus layer creating a physical barrier comprising the epithelium and the mucus layer (**Figure 1A**) which makes them suitable for gastrointestinal infection studies [114-116].

3.1.2 Metabolic labeling of mucins

Pathogenic invasion of the epithelium can modify mucin structure and synthesis by the pathogen per se and/or components of the immune response to infection [2]. The effect of A/E pathogens and cytokines on the production and secretion of mucins is not fully explored. Mucins are highly post-translationally regulated due to the high level of glycosylation which often goes undetected when measuring mucin mRNA levels and thus do not reflect mucin levels or mucus thickness. Therefore, in this thesis, we use the azido-glycan incorporation method to label mucin *O*-glycans and study the later stages of mucin biosynthesis in response to infection and/or cytokine stimulation. The *N*-acetylgalactosamine (GalNAc) analogue GalNAz incorporates into glycosylated mucins and is visualized by “click” chemistry based on attachment of a fluorescent alkyne to GalNAz [117, 118]. GalNAz was

injected intraperitoneally in mice or added basolaterally to *in vitro* mucosal surfaces and the incorporation of the metabolic label into *O*-glycans was detected by fluorescence staining using the reaction mix tetramethylrhodamine (TAMRA) (**Figure 1 B1**). GalNAz incorporation into newly synthesized mucins was visible at the supranuclear compartment within an hour in the *in vitro* model. This is in line with the metabolic labeling observed in *in vitro* mucosal surfaces and in the gastrointestinal tract with release of the labeled mucins into the apical surface or lumen in 6 - 8 h [10, 79, 80, 116]. Analysis of the quantity and cellular location of labeled mucins should be carried out while they are still located inside the cell before reaching the lumen or secreted apically in the *in vitro* model, due to that the mucus layer sometimes is lost, partially lost or damaged during processing, which can lead to artefacts. Therefore, the *in vitro* and *in vivo* models were analyzed at 2 h and 3 h post GalNAz addition/injection, because the labeled mucins are still inside the cells (**Figure 1 B1 and B2**) although an increase in mucin turnover and secretion occur in response to infection and/or treatment (Paper III). Changes in glycosylation can occur during infection and inflammatory responses [119]. To ensure changes in label intensity are not due to changes in incorporation of GalNAc during mucin glycosylation, we routinely couple the GalNAz method with mass spectrometric analysis of the glycans. To investigate if Muc2 contributes to the increased mucus thickness, goblet cells were scored separately from other epithelial cells to exclude the membrane-bound mucins Muc4, Muc13 and Muc17 which incorporate the metabolic label faster than the secretion of large multimeric mucins into the lumen [79].

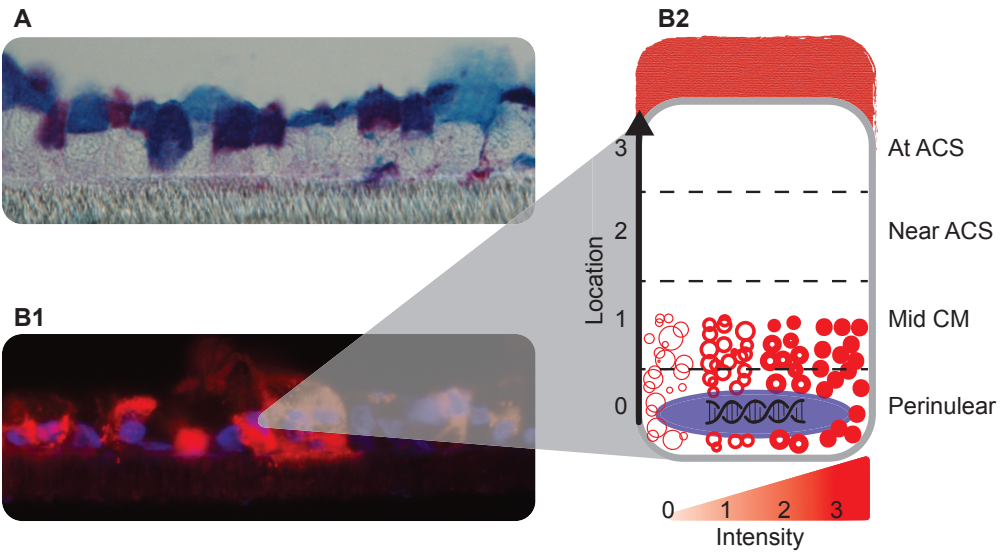

Figure 1. Mucus producing *in vitro* mucosal surfaces. A) AB/PAS stained *in vitro* mucosal surface at day 34 post confluency. B1) Incorporated GalNAz to *in vitro* mucosal surfaces 2 h post addition of label, TAMRA (red) and DAPI (blue) captured at 60x. B2) An illustration of the semi-quantification of the location (vertical black arrow) and intensity (bottom red gradient) of incorporated GalNAz in goblet cells. Each location (horizontal lines) receives a score from 0-3 and is combined with fluorescence intensity (bottom red gradient). Apical cell surface (ACS), cytoplasm (CM).

4 RESULTS

4.1 IL-4 protects mitochondrial function during infection (Paper I)

Mitochondria supply the majority of ATP or energy for eukaryotic cells; however, disturbances in mitochondrial function lead to apoptosis [120]. The T3SS used by A/E bacteria aid in the translocation of Map and EspF, these mitochondria-specific effector proteins are involved in disruption of cellular homeostasis [42, 44, 120]. Commensal and pathogenic bacterial products, and host derived pro-inflammatory cytokines (TFN-α and IFN-γ) interfere with mitochondrial functions in inflammatory bowel disease (IBD) [121]. We examined mitochondrial activity in the colon of IFN-$\gamma^{-/-}$ mice as (Paper III) they possess a different cytokine profile without IFN-$\gamma^{-/-}$ and more anti-inflammatory cytokines that proved beneficial for goblet cell function. We found that IFN-$\gamma^{-/-}$ mice were protected against factors involved in mitochondrial malfunction when bacterial density reached its peak. To prove that mitochondrial dysfunction is cytokine driven we carried out an *in vitro* infection assay with pro- and/or anti-inflammatory cytokine treatment. We treated the *in vitro* model with TNF-α or IFN-γ with and without infection, individually or in combination, and found a loss of mitochondrial function associated with both the pathogen and pro-inflammatory cytokines matching the *in vivo* experiment (**Figure 2 C3 and C5**). The addition of cytokines that were found to be upregulated during clearance namely, IL-4 and to some extent IL-6, to the cytokine mixture reversed these aspects indicating that IL-4 protects mitochondrial function and that cytokines rather than the pathogen *per se* are responsible for this (**Figure 2 C7**).

4.2 VIP protects mitochondrial function during infection (Paper II)

VIP is a neuropeptide expressed in the colon. It is believed to possess anti-inflammatory properties [106], inhibit pro-inflammatory immune responses [122],

and regulate intestinal ion and fluid secretion [104]. Intestinal inflammation increases the density of VIP in the colonic mucosa and mice deficient in VIP show loss of goblet cells that may be restored upon VIP treatment [108, 109]. We investigated whether VIP can protect mitochondrial dysfunction caused by A/E pathogens (Paper I) to restore the mucus layer and alleviate colitis. The level of VIP in WT mice showed a gradual decline during the course of infection whereas IFN-$\gamma^{-/-}$ mice were less affected suggesting that the cytokine environment rather than the pathogen density is the cause. *In vitro* VIP treatment alleviated the effect of added pro-inflammatory cytokines in the presence or absence of *C. rodentium*. Moreover, *in vivo* VIP treatment from day 5-10 post-infection (PI) alleviated the negative effect of *C. rodentium* infection on parameters associated with mitochondrial function and apoptosis (**Figure 2 C1, C2 and C8**). *C. rodentium* density at the epithelium tended to be reduced in all VIP treatment regimens used in this study. However, VIP treatment did not alleviate colitis and had negative effects on the health of the mice, especially with some treatment regimens. Possibly, one reason the anti-inflammatory properties of VIP did not promote faster clearance of the pathogen could be that IFN-γ is involved in the removal of *C. rodentium* [91] or that the signaling events from the mitochondria released during mitochondrial stress and apoptosis such as mtDNA and downstream TLR signaling is vital for proper organization of the defense.

4.3 IL-4 increases mucin production and reduces *C. rodentium* density at the epithelial surface (Paper III)

Adherent mucus layer thickness varies during the course of *C. rodentium* infection, where early colonization (day 4) and peak of bacterial density (day 10) in the colon results in a thin inner mucus layer followed by an increase in thickness during clearance of infection (day 14-19) [7]. There is compelling evidence suggesting that IFN-γ, amongst other pro-inflammatory mediated cytokines (IL-12 and TNF-α), play a role in host defense against *C. rodentium* infection [123]. Mice deficient in IFN-γ (IFN-$\gamma^{-/-}$) had a thicker mucus layer and increased amount of stored mucin in the tissue at day 10 PI. To find out which cytokines are involved in altering the mucus layer thickness in WT and IFN-$\gamma^{-/-}$ mice, we examined cytokine gene expression in

both genotypes during thickening of the mucus layer i.e. either day 10 PI for IFN-$\gamma^{-/-}$,or day 14-19 PI in WT mice. A selection of cytokines from the pro-/anti-inflammatory immune response expressed in the array were tested on *in vitro* mucosal surfaces for their ability to stimulate mucus secretion and goblet cell density either alone or combined, and with or without a pathogen (**Figure 2 C1-C7**). We found that treatment with pro-inflammatory cytokines (IFN-γ, TNF-α) decreased *in vitro* muin production when added alone or in combination with infection (**Figure 2 C3 and C5**). Conversely, treatment with anti-inflammatory cytokines particularly IL-4 enhanced mucin production and transport speed through the cell, especially when combined with infection, in addition to protecting goblet cells affected by pro-inflammatory cytokines in the presence of bacteria (**Figure 2 C4, C6 and C7**). To investigate the production rate and turnover of mucins of the *in vitro* model we treated the cells with pro- and anti-inflammatory cytokines with and without infection and harvested them at 2 h and 5 h post GalNAz exposure (**Figure 1 B1 and B2**). Pro-inflammatory cytokine treatment reduced mucin turnover rate while anti-inflammatory cytokine treatment enhanced the turnover of mucin especially in the presence of a pathogen compared to non-treated controls regardless of infection (**Figure 2 C1, C3, C4, and C6**). These results indicate that the pathogen and cytokines together alter goblet cells, mucin production and secretion, and the mucus layer dynamics during infection. Flow cytometry analysis revealed that IL-4^{+} T cells in the colon of *C. rodentium* infected WT mice contribute to the elevated IL-4 expression leading to an increase in mucin production and secretion. Furthermore, gene expression data from distal colon samples of mice from day 10 PI for IFN-$\gamma^{-/-}$, or day 14-19 PI in WT mice revealed that the IL-4, IL-4 receptor α (IL-4Rα), Stat6, SAM pointed domain containing ETS transcription factor (Spdef) pathway most likely contributed to the *in vivo* increase in goblet cells, mucus production and secretion. We manipulated the IL-4/Stat6 pathway in *C. rodentium* infected WT mice when bacterial density in the colon reached its peak and found that IL-4 increased mucus thickness and quality, reduced *C. rodentium* in contact with the mucosa, and decreased colitis levels.

4.4 Fpr2 offers protection against *C. rodentium* colonization and pathogen dissemination (Paper IV)

Recruitment of immune cells to infected tissues relies on chemoattractants released in the local environment during pathogen invasion. Fpr2 is well known for its ability to bind N-formyl peptides, originating from damaged host cell mitochondria or the pathogen, for neutrophil recruitment. Furthermore, the lack Fprs affect wound healing and susceptibility to DSS-induced colitis [64, 124]. As Fpr2 has been shown to respond to cytokines [65] and bacterial products [66] we hypothesized that mice deficient in Fpr2 ($Fpr2^{-/-}$) would be more susceptible to *C. rodentium* infection. Furthermore, several Fpr ligands are involved in maintaining tissue homeostasis, including the intestinal epithelium [125, 126]. We therefore hypothesized that Fpr2 was important for maintaining the barrier functions of the epithelium, including the mucus layer. In our study, we used a conventional *C. rodentium* dose to investigate the effect of infection on the localization of the Fpr2 protein on colonic epithelial cells. We found that Fpr2 localization increased during infection in WT mice compared to non-infected controls. Using the same bacterial dose to infect $Fpr2^{-/-}$ mice showed that recovery from infection was somewhat delayed compared to infected WT mice. This is in line with previous studies demonstrating defects in intestinal wound repair in the absence of Fprs [126]. We challenged the mice using a low *C. rodentium* inoculum dose as decreased barrier functions would lead to greater colonization if $Fpr2^{-/-}$ mice are more susceptible to infection. Indeed, all $Fpr2^{-/-}$ mice became colonized versus a fraction of the WT mice, showing bacterial translocation to the spleen and liver and higher colitis score in $Fpr2^{-/-}$ mice. Moreover, substantial pathogen invasion of the colonic epithelium was found in $Fpr2^{-/-}$ mice coinciding with a disturbed inner mucus layer. Histological findings based on AB/PAS staining show that more mucus is present along the crypts of $Fpr2^{-/-}$ mice. Nevertheless, metabolic labelling experiments demonstrated that the speed of transport of mucins was slower in $Fpr2^{-/-}$ mice, which was further supported by that the mucin glycans had increased size and sialylation pointing toward altered mucin biosynthesis that may be influenced by the difference in the cytokine profile between $Fpr2^{-/-}$ and WT mice. *In vitro* mucosal surfaces infected with *C. rodentium* and EPEC confirmed

increased translocation of bacteria across the *in vitro* mucosal surfaces treated with various Fpr2 inhibitors and confirming the *in vivo* results of bacterial translocation to extraintestinal organs. This suggests that FPR2 on epithelial cells and not only on circulating immune cells are important for the protection against infection.

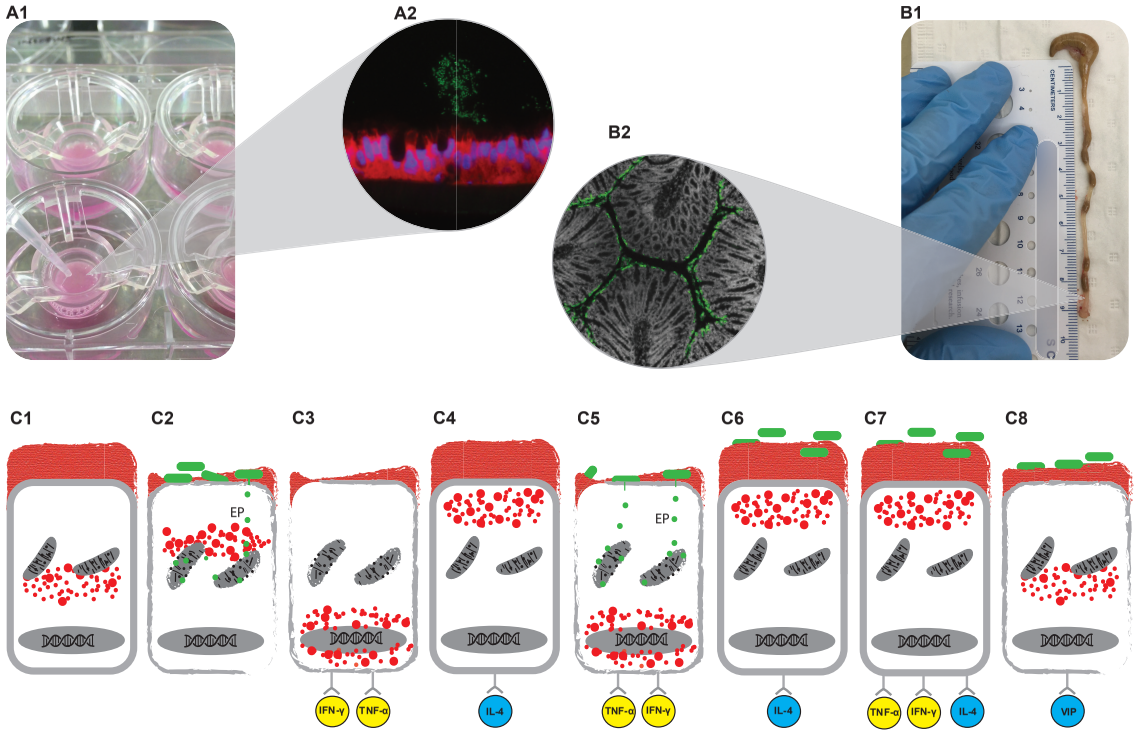

Figure 2. **Effect of infection, cytokines and VIP on mouse distal colon and *in vitro* mucosal surfaces**. A1) Image of EPEC infected *in vitro* mucosal surface covered by a sheet of mucus. A2) The *in vitro* mucosal surface showing EPEC (green) separated from the cell surface (red) by the mucus barrier, nuclei (blue). B1) *C. rodentium* infected mouse colon tissue from caecum (top) to distal colon (bottom). B2 *C. rodentium* (green) infected distal colon tissue (nuclei, grey). C1 – C8) An illustration of the effect of infection, cytokines and VIP on *in vivo* and *in vitro* mucus production, barrier and mitochondrial function. Images were captured at 40x (A2) and 10x (B2). A/E effector proteins (EP).

5 DISCUSSION

5.1 IL-4 and VIP protects mitochondrial function during infection (Paper I and II)

It is well known that mitochondria are affected by inflammation, however less is known about the effect of cytokines and A/E infection on mitochondrial function in colonic epithelial cells. Our results from the *in vivo* experiment show that in WT mice *C. rodentium* are responsible for factors involved in mitochondrial dysfunction from which IFN-y$^{-/-}$ mice were protected against. Furthermore, during peak of bacterial load WT mice elicited a cellular pro-inflammatory response whilst IFN-y$^{-/-}$ mice presented an anti-inflammatory type of response to infection similar to WT mice during clearance. This was later confirmed using *C. rodentium*-infected *in vitro* mucosal surfaces based on cytokine genes upregulated during peak of bacterial load in IFN-y$^{-/-}$ mice or clearance in WT mice. Signature cytokines of pro- (TNF-a, IFN-y) and anti-inflammatory (IL-4, IL-13) responses were introduced either separately or in combination, and the results indicated that mitochondrial dysfunction is indeed cytokine driven. Moreover, anti-inflammatory cytokines enhanced mitochondrial- and goblet cell function in general and IL-4 reversed the negative effects of the pro-inflammatory cytokines on mitochondrial function.

The neuropeptide VIP regulates immune responses [122], intestinal ion and fluid secretion [104], and goblet cell density [108]. Therefore, we investigated whether the anti-inflammatory action of VIP could protect the mitochondria, alleviate colitis symptoms, and restore the mucus barrier during infection. We found that the loss of VIP in the colon of infected mice was cytokine driven and treatment with VIP restored mitochondrial function. However, although colitis did not improve, treatment with VIP reduced the density of *C. rodentium* at the epithelial layer possibly though recruitment of immune cells by IL-8 secretion [127] or an increase in mucus secretion. Although pro-inflammatory cytokines are responsible for intestinal damage [128] an anti-

inflammatory response mediated by the action of VIP may regulate mucus secretion and tissue repair, but might not be sufficient for pathogen clearance.

5.2 IL-4 induces rapid mucin secretion, increases mucus thickness and decreases pathogen colonization of the epithelium (Paper III)

We investigated the effect of pathogens and the cytokine environment on mucus layer thickness based on an earlier study [7] showing that this layer is dynamically regulated during the course of infection with thickening during clearance of the pathogen. We found that the cytokine environment increases the production and transport speed of mucins through goblet cells during clearance of *C. rodentium* infection. This was confirmed in IFN-$\gamma^{-/-}$ mice assessed during peak of bacterial load, which showed a thick mucus layer, engorged goblet cells and a cytokine environment similar to WT mice during the clearance phase. Mucin mRNA levels, although widely used in the field [129,130], do not take into account the post-translational modifications such as glycosylation affecting the structure, rate and turnover of mucins [131]. In this study, the difference in mucin mRNA levels during thickening of the mucus layer in both genotypes could not explain the increase in mucus thickness. However, the addition of IL-4 and infection increase mucin production and transport speed in *in vitro* mucosal surfaces, whereas pro-inflammatory cytokines had an inhibitory effect. During clearance of the pathogen in WT mice, when the cytokine profile switches from a pro-inflammatory response to one that is more similar to anti-inflammatory response, we found an increase in mucin production and transport speed probably owing to the increase in IL-4 and IL-13 at that time-point. The removal of pathogens from the tissue in nematode infections requires an increase in mucus secretion through IL-13 [132], whereas in our study, IL-4 is regulating mucin production. This discrepancy might be due to the combined action of the pathogen and the host immune system in regulating mucins during infection. In addition, by manipulating the IL-4/IL-4Rα/Stat6/Spdef pathway we could alter the properties of the mucus layer and pathogen localization relative to the epithelium *in vivo*. This pathway has also been shown to induce airway mucus production through binding of IL-13 to IL-4Rα [133, 134]. The importance of Muc2 in protecting mice against infection and binding to *C. rodentium* [73-75]

adds more strength to our observation of IL-4 as a potential therapeutic cytokine that increases mucus layer thickness and quality and decreases colitis and pathogen density at the epithelial surface.

5.3 Fpr2 deficient mice are more susceptible to *C. rodentium* colonization and infection (Paper IV)

In this study, Fpr2-deficient mice were found to be more susceptible to colonization by *C. rodentium* due to a decrease in colonic barrier function. In line with previous reports, the lack of Fpr2 or their biding proteins rendered mice more susceptible to DSS-induced colitis, aggravated histopathology and prolonged recovery time compared to WT mice [64, 135]. Challenging immunocompetent WT mice with a conventional inoculum dose ($>10^8$ CFU) of *C. rodentium* produces characteristic histopathological lesions of A/E bacteria and the pathogen is cleared within 3-4 weeks post infection [25]. Susceptibility to *C. rodentium* infection has been investigated in various mouse strains using a conventional dose reporting on rapid and extensive colonization and mortality [136]. Furthermore, knock-out of genes involved in the immune response against *C. rodentium* result in extensive tissue damage and/or persistent infection [137, 138 92, 139]. In this study, $Fpr2^{-/-}$ mice infected with a conventional dose showed prolonged recovery from colitis symptoms which point towards, and are in line with, a defect in epithelial wound repair as a decrease in the anti-inflammatory response has been observed in $Fpr2^{-/-}$ mice following DSS-induced colitis [64].

However, a low *C. rodentium* inoculum dose resulted in heavy colonization of the colonic epithelium early after infection. Slower mucin production and a defective inner mucus layer may account for the increased colonization observed in these mice early during infection. A defective inner mucus layer may be the result of an altered microflora caused by changes in the glycosylation pattern of mucins, an increase in mucin degradation by the microflora exceeding mucus replenishment, or the negative charge of *O*-glycans increasing inter-mucin repulsion affecting the organization of the inner mucus layer. As discussed in Paper III, cytokines regulate mucin production and secretion, mucus thickness and quality and pathogen localization in the colonic epithelium. The lower level of TNF-a, IL-2, IL-6

and IL-13 in Fpr2$^{-/-}$ mice during early infection might alter mucin production, early host defense, mucosal inflammation and pathogen dissemination [92, 132, 140]. Agonist stimulation of Fpr2 has been shown to reverse epithelial permeability in DSS-induced colitis [141] thus, epithelial permeability may account for the increase in dissemination of *C. rodentium* to the spleen. Fpr2 stimulation of polymorphonuclear cells trigger their recruitment to inflamed tissues [142, 143], which in the absence of Fpr2 may be poorly regulated, hence resulting in higher *C. rodentium* density in the spleen early during infection preceding *C. rodentium* specific IgG production important for pathogen elimination [96]. The role of epithelial FPR2 in limiting bacterial dissemination was confirmed in human *in vitro* mucosal surfaces showing that FPR2 inhibitor treatment increased EPEC translocation across the mucosal surfaces. In summary, the activation of Fpr2/FPR2 may protect against infection-induced mucosal injury.

6 CONCLUSION

In this thesis we investigated the changes in mitochondrial function, mucin production and the mucus layer organization in the mouse colon during infection with *C. rodentium*. The dynamic regulation of the colonic mucus layer during infection was governed by the cytokine response elicited during different time-points of *C. rodentium* infection. By supplementing the host's cytokine response to infection, we could alter mucin production, mitochondrial and goblet cell function, and pathogen localization. The results were verified using *in vitro* mucosal surfaces infected with A/E pathogens and ETEC.

- The dynamic changes in mucin production and mucus thickness during *C. rodentium* infection in mice are strongly affected by changes in the cytokine environment

- Pathogens and anti-inflammatory cytokines, especially IL-4, accelerated mucin production and transport through the cells

- *In vivo* IL-4 treatment starting at height of infection increase mucin production, mucus thickness and organization, reduce penetrability of the mucus layer and *C. rodentium* density at the epithelium, and alleviates colitis symptoms. The anti-inflammatory cytokine profile in IFN-γ deficient mice likely protected mitochondrial function

- *In vitro* IL-4 treatment increase mucin production, protects goblet cells and mitochondrial function from the damaging effect caused by infection and pro-inflammatory cytokines

- Lack of Fpr2 leads to decreased barrier functions against *C. rodentium* and EPEC and mice lacking FPR2 have decreased mucin production

- Both *in vivo* and *in vitro* VIP treatment improved mitochondrial function and restored the damaging effect of the pathogen and pro-inflammatory cytokines, however, IL-4 treatment was superior to VIP treatment in many ways

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Shabab: They say times flies when you are having fun. 20+ years of friendship and still going strong, it is longer than the average relationship.
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