Host and Pathogen Regulation of Intestinal Epithelial Inflammatory Responses During Bacterial Infections

by

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Abstract
The attaching and effacing (A/E) bacterial pathogen enteropathogenic *Escherichia coli* (EPEC), targets the intestinal epithelial cells (IEC) lining the gastrointestinal tract, causing severe diarrhea and potentially death. Although IEC express Toll like receptors (TLRs), they are hypo-responsive to most bacterial products, thereby preventing overt inflammatory responses against commensal bacteria. Single Ig IL-1-Related Receptor (SIGIRR) is a negative regulator of interleukin (IL)-1 and TLR expressed by IECs. Its expression by IEC may limit their ability to respond to invading pathogens, potentially increasing host susceptibility to infection. To address whether SIGIRR expression influences host defense against enteric pathogens, *Sigirr* deficient (-/-) mice were infected with the mouse adapted A/E pathogen *Citrobacter rodentium*. *Sigirr* -/- mice responded with accelerated IEC proliferation, and strong pro-inflammatory and antimicrobial responses in this study. Yet, they were highly susceptible to infection. Exaggerated IEC response of *Sigirr* -/- mice were primarily dependent on IL-1R signaling leading to the rapid and dramatic loss of competing commensal microbes from the infected intestine. Thus, SIGIRR promotes commensal-based resistance to pathogen colonization despite limiting IEC responses to infection. Besides the host, A/E pathogens also actively suppress IEC inflammatory and anti-microbial responses using a type 3 secretion system to deliver bacterial effector proteins into infected IEC. To identify these effector(s), I tested an array of EPEC mutants and identified that non-LEE encoded effector (Nle)C suppressed the release of the chemokine IL-8 from infected IEC in *vitro*. NleC localized to EPEC-induced pedestals and inhibited both NF-κB and p38MAP kinase activation. Comparison between mice infected by Δ*nleC* to wildtype *C. rodentium* demonstrated that loss of NleC did not impact pathogen burdens but did result in more severe colitis. Furthermore, Δ*nleC* compared to wildtype *C. rodentium* induced significantly greater
chemokine responses. Thus, innate IEC responses are actively suppressed by the host in an attempt to prevent enteric infections. However, once A/E pathogens succeed in infecting their host, they try to suppress IEC responses to prolong the infection. These studies highlight the importance of IEC as the key player in controlling host susceptibility to pathogens and in maintaining a mutualistic relationship with the intestinal commensal microbiota.
Preface

Chapter 2
A version of this chapter has been submitted for publication. I designed and conducted 80% of the studies described in the chapter, analyzed all the data and wrote the manuscript under the supervision of Dr. B. A. Vallance. Dr. Xiaoxia Li and Dr. Muhammet F. Gulen generated the *Sigirr* -/- mice and provided them as well as the MyD88 flox mice. Two graduate students, Mr Justin M Chan and Ms Ganive Bhinder performed the histology pathology scoring leading to Figure 2.1D, 2.2D, 2.6D. Ms Emily Yi Shan Yu and Ms Lara Brewster, both undergraduate students under my supervision, performed the bacterial enumeration and real time quantitative polymerase chain reaction studies described in Figure 2.4, 2.5D 2.8B, 2.9D and 2.12. Dr. Kelly McNagny and Dr. Michael Hughes generated bone marrow chimeric mice leading to Figure 2.6. Dr. Vijay Mormapudi performed that Western blot shown in Figure 2.9B. Dr. Deanna Gibson assisted in the experimental design of the commensal analysis shown in Figure 2.12.

**Ho Pan Sham**, Emily Yi Shan Yu, Muhammet F. Gulen, Justin M. Chan, Ganive Bhinder, Deanna L. Gibson, Vijay Mormapudi, Michael Hughes, Kelly McNagny, Xiaoxia Li, and Bruce A. Vallance. Single Immunoglobulin IL-1R related molecule (SIGIRR) is a critical factor in host defense against enteric pathogens by promoting mutualism with commensal bacteria

Chapter 3
I was the primary contributor to this work, and developed the experimental plan, carried out the majority of experiments, analyzed results, and prepared all the figures. This work was published in the journal of Infection and Immunity. Dr. B. A. Vallance aided in the experimental design and supervised this research. Dr. Stephanie Shames performed the *in vitro* studies leading to Figure 3.3B. Ms. Caixia Ma, Dr. Mathew Croxen and Dr. Wanyin Deng generated the bacterial mutant and complemented strains used in this study. Dr.
Mohammed Khan assisted in the experimental design and Dr. Mark Wickham contributed data shown in Figure 3.7. Infection and Immunity has granted permission to reproduce the manuscript in full as part of the thesis.


Ethics approval:

Animal work was performed under the approval of the UBC Animal Care and Use Committee (UBC protocol numbers: A07-0084, A07-0089, A09-0604, A11-0290, A11-0253)
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<td>±</td>
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<td>A/E</td>
<td>Attach and Effacing</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>HA</td>
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<td>Myeloid differentiation primary response gene (88)</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<td>NF-κB</td>
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<td>SIGIRR</td>
<td>Single Immunoglobulin Interleukin-1 related receptor</td>
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<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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To my parents and my family who think the world of me and believe I can do anything
1 Chapter: Introduction

It has been said that “we are more bacteria than we are human”. Indeed, there are trillions of bacteria residing within our gastrointestinal (GI) tracts, far outnumbering the human cells that make up our bodies [1]. Our relationship with the bacteria in our GI tracts is vastly different from how our bodies respond to bacteria within organs that are relatively sterile. In most sites within our body, the presence of bacteria leads to a rapid and strong inflammatory and anti-microbial response. In contrast, our intestines co-exist in relative harmony with most of our resident intestinal microbes that are collectively known as our commensal bacteria or the commensal microbiota [2]. In fact, this cohabitation between microbes and mammals has evolved over time and developed into a mutualistic relationship. The commensal microbiota enhance the digestive efficiency of their hosts, promote proper immune system development within the GI tract and even compete with invading bacterial pathogens to limit their ability to colonize and infect their hosts [3,4,5,6]. In return, the host GI tract provides a protected, nutrient rich environment for the commensal microbes [7,8]. In this complex and dynamic relationship, there is some level of crosstalk between the commensal microbiota and their hosts, with much of this communication involving the cells lining the intestine, namely the intestinal epithelial cells (IEC) [9].

The physical barrier provided by the intestinal epithelium separates the contents of the intestinal lumen, including the commensal microbiota, from the rest of the body, and most critically, from the systemic immune system [10,11,12]. This physical barrier undergoes significant challenges from a variety of noxious luminal stimuli, including invading microorganisms. Not surprisingly, the IEC layer plays a critical role in initiating and
regulating the immunological balance within the intestine [13]. One of the biggest challenges for IEC is their role in ensuring the development of a rapid and strong immune response to pathogenic threats, while maintaining tolerance to commensal microbes under physiological conditions. In comparison with other organs, the presence of commensal microbes promotes a heightened inflammatory tone within the GI tract, known as physiological inflammation [14]. This refers to the GI tract expressing a high baseline level of inflammatory cytokines and containing a large number of resident immune and inflammatory cells, as compared to other tissues. Rather than overt tissue damage and inflammation, this increased inflammatory tone enhances the physiological function such as aiding host defense and enhancing tissue repair of the GI tract [15]. In contrast, infection by bacterial pathogens triggers significant inflammatory responses within IEC, such as the release of antimicrobial peptides and the production of chemokines and cytokines that promote the further recruitment and overt activation of professional immune cells to clear the infection [8,10,16]. It has been suggested that innate receptors such as Toll like receptors (TLR) that recognize conserved microbial components such as lipopolysaccharide or flagellin, assist IEC in distinguishing between commensal microbes and pathogens [10,11]. At present however, the specific mechanisms by which IEC distinguish commensal from pathogenic bacteria remain unclear.

Nonetheless, it is essential for the host to be able to develop an effective inflammatory response in order to fight and clear pathogens. However, the development of excessive or prolonged inflammation can disrupt mucosal homeostasis and can lead to chronic intestinal pathologies such as Inflammatory Bowel Disease (IBD) [17]. In order to prevent such exaggerated inflammation, the IEC layer is known to be hypo-responsive to most bacterial
ligands under steady state conditions, presumably to prevent the development of overt inflammation in response to the nearby commensal bacteria [18]. While the exact mechanisms by which this hypo-responsiveness is achieved are not entirely clear, it appears to involve various suppressive mechanisms including tight control over the expression of innate receptors and the use of negative regulators to attenuate/dampen immune responses [19,20,21,22].

Despite their hypo-responsiveness, IEC as well as the cells within the underlying mucosa are capable of developing significant inflammatory and antimicrobial responses in response to infection by bacterial pathogens [23,24]. As a result, all successful pathogens have had to develop an array of mechanisms to subvert host defenses including inflammatory and immune responses [25]. Enteric bacterial pathogens such as enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *Escherichia coli* (EHEC) inject virulence associated proteins (effectors) into host cells in order to hijack their signalling pathways, including those that control innate immunity and other defense barriers within the gut [26,27]. Ultimately the resulting host response to enteric bacterial pathogens is determined not only by host factors, but also by the pathogen. The goal of this “tug-of-war” between host and pathogen is aimed at controlling the inflammatory status and function of the IEC. In order to fully explain how my research has aided in our understanding of how these interactions play out, the structure and functions of the different regions that make up the mammalian GI tract will be explored in the following sub-chapters.
1.1 The Gastrointestinal (GI) tract

The mammalian alimentary canal, also known as the GI tract, refers to the tubular passage that extends from the mouth to the anus [28,29]. The GI tract mediates both the digestion and the absorption of food as well as the elimination of residual wastes. The adult human GI tract totals about 9 meters (m) in length with a total mucosal surface area of 200-300 m² representing the largest site in the body in contact with the external environment. The GI tract can be divided into upper and lower regions, with the upper GI tract consisting of the mouth, pharynx, esophagus and stomach while the lower GI tract consists of the small intestine (duodenum, jejunum and ileum), large intestine (appendix, cecum, colon and rectum) and anus. Food travels in an aboral direction from the mouth towards the stomach through the esophagus. The stomach churns and mixes ingested food with digestive juices creating liquid chyme. The digestive juices contain hydrochloric acid that reacts with pepsinogen, which is then converted into pepsin that continues digesting food proteins into smaller peptides. The small intestine receives the acidic chyme and further digests food material, subsequently absorbs many of the products of the digestive process. At around 7 m in length, the small intestine is the longest region of the GI tract. The small intestine is further subdivided into three sections, the duodenum, jejunum and ileum. Although these regions are similar histologically, their minor differences permit their histological identification.

From the duodenum to the ileum, the number of goblet cells, a type of secretory epithelial cell, increases. Furthermore, the ileum houses permanent clusters of organized lymphoid nodules, known as Peyer’s patches [29]. The duodenum receives digestive enzymes and an
alkaline buffer from the pancreas to aid in digestion and neutralization of the acidic chyme. Additionally the duodenum receives bile from the liver to aid in the digestion of fat into fat droplets. Following the digestion of food, the absorption of nutrients takes place within the small intestine. Through evolution, the luminal surface of the small intestine has been modified to increase its surface area in order to improve the efficiency of nutrient intake. The entire small intestinal mucosal lining is covered by finger like projections called villi which increase the luminal surface area of the small intestine by 10-fold. Furthermore the lining of each villus is made of absorptive IEC that are topped with microscopic projections called microvilli. Microvilli further increase the surface area of the small intestinal epithelium by another 20-fold.

Finally the digested chyme travels to the large intestine, where water, electrolytes and gases are absorbed. After chyme is compacted into feces through the removal of water; it leaves the body through the opening of the rectum, also known as the anus. Once food is ingested, it takes about six to eight hours to pass through the stomach and the small intestine. It can take up to another twenty-four hours before the undigested food material (packaged as feces/stool) is excreted from the body. The broad range of physiological conditions and functions in the GI tract create numerous distinct niches for microorganisms to colonize [30]. The low pH in the stomach limits microbial numbers, in fact only a few bacterial species are able to colonize the stomach. The resident microbial communities become larger and more diverse as one moves down the GI tract. In the small intestine, approximately $10^7$ bacteria/gram of luminal material can be detected [12,30]. Within the large intestine, the relatively slower transit time and the readily available supply of nutrients from undigested food particles allow for the
highest density and abundance of microorganisms [31]. Overall, up to $10^{12}$ bacteria/gram are commonly detected in human feces [32,33]. Although the GI tract is long and encompasses different sections, it has a uniform general histological structure with relatively modest differences that reflect regional functional specializations. Since the description of each section lies beyond the scope of this thesis, I will focus my attention on the IEC and mucosal layer of the large intestine, which is most pertinent to my research.

1.2 The large intestine

The human large intestine, comprised of the cecum, colon, rectum and anus, is approximately 1.5 m long [28]. The large intestine mediates the absorption of water, electrolytes, and gases as well as aids in the compaction and elimination of feces [29]. The cecum and colon are histologically similar, and both are home to many anaerobic bacteria residing within the intestinal lumen. These anaerobic bacteria aid in breaking down some types of indigestible food material, while also producing vitamin K and essential amino acids for the host [4]. The colonic epithelium consists of a single sheet of columnar epithelial cells that does not form villi (as seen in the small intestine). It is instead folded into finger-like invaginations that are surrounded by the lamina propria to form a functional unit, called the crypts of Lieberükhn [28,34]. IEC that make up this monolayer are interconnected via tight junctions, which regulate and maintain barrier permeability, acting as a fence to prevent large luminal antigens or microbes from reaching the underlying lamina propria [35]. The lamina propria is where professional immune cells such as dendritic cells, lymphocytes and macrophages reside (Figure 1.1). Aside from the cells distributed throughout the lamina propria, some of these immune cells cluster together to form lymphoid follicles, also known as gut-associated
lymphoid tissues (GALT). Studies indicate that the IEC also function as a communicator between the luminal commensal microbes and the subepithelial immune system [10,13]. For instance, contact with commensal microbes has been shown to induce IEC to release cytokines such as IL-8, TNF-α, MCP-1 and IL-6 that specifically regulate the development of immune cells found within the lamina propria [12,36,37]. A competent and functional IEC barrier therefore ensures protection against harmful agents. However, studies indicate the IEC also allow small amounts of luminal antigens (from food and commensal microbes) to cross the epithelial barrier, in order to interact with and to educate the mucosal immune system [8,35,38,39].

![Figure 1.1: The colonic mucosa](Image)

Schematic diagram of the colonic mucosa. Mucus released by goblet cells covers the surface of the intestinal epithelium limiting commensal microbe contact with the IEC. Beneath the mucus layer is a single layer of epithelial cells that acts as a physical barrier, preventing luminal factors from contacting the underlying immune cells like macrophages, neutrophils and dendritic cells that are found within the lamina propria. (Image reproduced from [40] with permission)
1.2.1 Intestinal epithelial lineages

Far from being a homogenous cell population, the intestinal epithelium is composed of several distinct cell types, each of which contributes to intestinal barrier function [34]. There are three major cell lineages within the colonic crypt epithelium: enterocytes, goblet cells, and endocrine cells [41,42]. These three terminally differentiated cell types are derived from multipotent stem cells located at the base of the crypts. As they undergo asymmetric division, the epithelial stem cells undergo self-renewal and generate a population of transient amplifying cells. As they migrate upward through the crypt, these transient cells differentiate into one of the three epithelial cell types of the colonic crypt. Rapid mitotic activity of the regenerative cells replaces the epithelial lining of the crypts and of the mucosal surface every 6-7 days [42]. Once epithelial cells of any type reach the top of the crypts they undergo a form of programmed cell death (anoikis) and are extruded into the intestinal lumen [43].

The most abundant cells in the colonic epithelium are the enterocytes. Like many of the other epithelial cells in the body, enterocytes have a polarized phenotype [34]. Enterocytes exhibit apical-basal polarity where the apical membrane faces the intestinal lumen and the basal membrane is oriented away from the lumen [44] (Figure 1.2). The apical side of the polarized enterocyte forms finger-like projections increasing the surface area of the cell to heighten efficiency of its absorption of water and other nutrients [29]. Besides their involvement in digestive functions and their role as a physical barrier, enterocytes also provide defense against luminal microbes by producing antimicrobial peptides such as β-defensins and cathelicidins [45,46].
Figure 1.2: Epithelial lineage in the large intestine
Schematic of the cellular organization of the large intestinal crypts. The proliferation zone is located in the bottom portion of the crypts and the upper half of the crypts consists of non-proliferating, terminally differentiated cells. In the small intestine, the base of the crypts also contain Paneth cells, which are absent in the large intestine.

The second most abundant epithelial cell type within the large intestine is the specialized secretory goblet cell [34]. Goblet cells increase in numbers from the proximal to the distal portion of the GI tract. The main function of goblet cells is to produce heavily glycosylated mucins that, once released apically, hydrate to form a mucus matrix that covers the lumen-facing surface of the intestinal epithelium. The mucus gel layer prevents large molecules from reaching/interacting with the epithelial surface, providing a second semi-permeable intestinal barrier, while allowing small molecules such as short-chain fatty acids (SCFA) to pass through to the IEC [4,47]. The IEC ultimately absorb these SCFA and use them as a source of energy. The mucus layer thus limits contact between the luminal microbial community and the underlying IEC, and most recently, our research showed it also helps flush intestinal contents and intruding microbes away from the IEC surface [48]. Aside from physically separating the IEC surface from luminal contents such as bacteria, the mucus layer
also provides a matrix that contains antimicrobial peptides and secreted immunoglobulin (Ig)A released by IEC and plasma cells, respectively, to further prevent bacterial penetration [23,49,50]. In the small intestine, another type of secretory cell, Paneth cells, are present and secrete antimicrobial peptides such as α and β defensins to help maintain the mucosal barrier [34,50,51]

The third type of epithelial cell is the endocrine cell, a relatively rare type of IEC that constitutes less than 1% of the epithelium [34]. Despite their rarity, they are the most diverse type of IEC with approximately fifteen different subtypes [42]. These endocrine cells produce hormones that influence gastrointestinal motility as well as other intestinal functions such as the secretion of gastric juices [52]. Together, the enterocytes, goblet cells and endocrine cells form a single layer of epithelial cells in the colon that separates the contents of the intestinal lumen from the underlying lamina propria. The space between these epithelial cells is sealed by tight junctions, which regulate the permeability of the intestinal barrier to prevent bacteria and undigested molecules from crossing.

1.2.2 Tight junctions and the intestinal epithelial barrier

IEC have a prominent role in the exchange of nutrients and fluids [53]. Therefore, they form a more permeable barrier compared to the epithelial barriers found at other sites such as the skin. The architecture of a selectively permeable epithelial barrier is essential to properly regulate fluid and electrolyte absorption and secretion, as well as prevent uncontrolled passage of antigens, such as undigested food, bacteria and their products across the epithelium [25,35,38,50,54,55]. The barrier properties of the IEC layer reflect the apical
junction complex, which links adjacent IEC together via tight junctions, adherens junctions, and desmosomes (Figure 1.3) [53].

![Figure 1.3: Epithelial structure and tight junctions](image)

Schematic diagram for the arrangement of junctions in polarized epithelial cells. The apical junction complex is formed from the tight junction, adherens junction and the desmosome. (Image reproduced from [56] and [53] with permission)

Tight junctions are the most apically located intercellular junctions found between IEC [54]. Tight junctions are multi-protein complexes composed of four primary groups of proteins: claudins, Zona-occludens (ZO), junction- adhesion molecules (JAM) and the Coxsackievirus and Adenovirus receptors (CAR) proteins [53,56]. While claudins are transmembrane proteins, ZO proteins act as anchors between claudins and actin filaments. These proteins
form the tight junction complex restricting the paracellular zone and forming a selective seal between cells [54]. As a result, tight junctions are able to limit solute flux across the paracellular space and are thus the principal determinants of mucosal permeability [55].

Supporting the tight junction complexes are adherens junctions and desmosomes [53]. The adherens junctions are located in the apical region of the epithelial cell, just below the tight junctions. They are connected to actin filaments in the cytoplasm and are thought to anchor each cell to its adjacent cells. The adherens junctions are made up of cadherin and catenin, providing strong adhesive bonds between adjacent cells. The adherens junctions are also important sites of intercellular communication. Adherens junctions are required for tight junction assembly, while desmosomes are located below adherens junctions and are connected to intermediate filaments in the cytoplasm. The major function of the desmosome is to anchor adjacent cells and to facilitate cell-to-cell communication and contact. Together, tight junctions, adherens junctions and desmosomes form the apical junction complex that allows for an effective and highly regulated epithelial barrier to prevent most microbes and other antigens from reaching the lamina propria and activating the resident immune cells.

1.3 Microorganisms within the intestine

1.3.1 The commensal microbes

As mentioned previously, the colon is heavily colonized with bacteria, reaching $10^{12}$ colony forming units (CFU) per gram of fecal material [3]. Sixty percent of the fecal mass in humans is made up of bacteria [57]. Collectively, the resident human commensal microbiota is composed of at least 1000 species, although its makeup is dominated by the phyla Bacteroidetes and Firmicutes [3,58,59]. Other groups such as the Actinobacteria and the
Proteobacteria represent a minority of the commensal microbiota. Microbial colonization of the human gut begins immediately after birth. Although the makeup of the microbiota dynamically changes during the first two years of life, it becomes relatively stable under physiological conditions afterwards [60,61]. Despite this stability, the microbiota of adult humans can still change in response to intestinal disturbances, such as those caused by enteric infections, antibiotics or significant dietary changes [62,63,64]. Repeated studies suggest the host and its commensal microbes have co-evolved as part of a mutualistic relationship. The resident commensal microbiota provides a large repertoire of digestive enzymes that are not encoded in the host genome [4,35]. Thereby, the commensal microbiota contributes to the digestion of dietary substances and the synthesis of essential food supplements, such as vitamin K. The commensal microbiota breaks down complex carbohydrates into SCFA [65]. SCFA serve as a source of energy and regulate the growth and differentiation of IEC, especially in the colon. The SCFA produced by the commensal microbiota contributes 5-10% of the total caloric requirements of humans. The microbiota also helps promote the proper development of the mucosal immune system [7,38,39,66] and prevents infections caused by enteric bacterial pathogens [5,67,68]. In turn, the host provides a stable environment for the microbes along with a steady nutrient supply.

1.3.2 Bacterial pathogens

In contrast to the beneficial actions of the commensal microbiota, enteric bacterial pathogens induce diarrheal diseases and disrupt the mutualistic relationship between the host and its commensal microbiota [1,14,24]. There are a number of enteric bacterial pathogens including species of Vibrio, Salmonella, Shigella and an array of pathogenic strains of Escherichia coli.
Pathogenic bacteria differ from commensal microbes in that they possess virulence factors that offer them a selective advantage in colonizing mammalian hosts, allowing them to invade host tissues and cause infectious disease [1]. Most enteric pathogens follow a defined strategy for infecting the GI tract, beginning with the expression of virulence factors that allow them to evade host defenses, followed by colonization of IEC as well as sometimes the underlying mucosa, and subsequent multiplication [24,25]. While infection on its own sometimes results in host tissue damage, it is the host response to these pathogens that causes much of the resulting disease. Much of my research focuses on a family of pathogens that colonizes the intestinal mucosal surface, namely the family of attaching and effacing (A/E) bacterial pathogens.

1.3.2.1 Attaching and effacing bacterial pathogens

Despite the array of innate immune host defense mechanisms found within the gut, and the stiff competition for nutrients from the resident microbiota, the GI tract is one of the most frequent sites of infection of mammals by pathogenic organisms [73]. Successful enteric pathogens, by definition, must be able to subvert or circumvent intestinal luminal defenses such as the intestinal mucus barrier in order to reach and infect the intestinal epithelium and underlying tissues. While the mechanisms by which enteric bacterial pathogens reach their target cells remain to be elucidated, on reaching the IEC, many pathogens subvert the resulting host inflammatory response by limiting the activation of innate receptors which recognize microbial components [24,74,75]. Like many enteric pathogens, A/E pathogens such as EPEC, EHEC and the related mouse pathogen *Citrobacter rodentium* also suppress
host inflammatory responses and other cellular functions by utilizing their virulence factors [26,76,77,78].

Attaching and effacing (A/E) bacterial pathogens are known for their ability to form A/E lesions on the surface of the IEC they infect [78,79]. A/E lesions are characterized by the bacteria’s ability to adhere to IEC, to destroy the underlying brush border microvilli, and to induce actin filled membranous protrusions, known as pedestals at the site of attachment. The A/E lesion-forming pathogens do not, in general, invade deeper layers of the mucosae or spread systemically, making them predominantly mucosal (luminal) pathogens. Two important human pathogens, EPEC and EHEC belong to this group of pathogens. EPEC is an important health threat across the world, causing watery diarrhea, particularly in young children in developing countries. As well it is a threat in nurseries and daycares in developed countries [69,80]. In contrast, EHEC is typically found in developed countries, as this microbe is often carried as a commensal microbe by cattle and other ruminants, however humans exposed to this pathogen following the consumption of undercooked/contaminated meat or contaminated water often suffer severe and bloody diarrhea [69]. EHEC is distinguished from EPEC by its production of the Shiga toxin. Shiga toxin can cause severe kidney damage leading to hemolytic uremic syndrome (HUS), a form of acute renal failure. The mechanisms by which EPEC and EHEC cause diarrhea are not fully understood, but appear to involve accelerated chloride secretion by IEC, as well as loss of absorptive area due to destruction of epithelial microvilli, and altered barrier function [80]. In most cases, the treatment for these infections is oral rehydration therapy to recover fluids and restore electrolyte balance.
1.3.2.2 EPEC pathogenesis and the subversion of host cellular processes

Among the A/E pathogens, the pathogenesis of EPEC is the best described. Following ingestion, and passage through the upper GI tract, EPEC eventually adhere to enterocytes, destroying their normal microvillus architecture. This process can be further broken down into three steps: i) initial adhesion, ii) protein translocation and iii) pedestal formation [69]. EPEC initially attaches to enterocytes using its bundle forming pili (BFP) that are coded on the EPEC adherence factor (EAF) plasmid. BFP acts like tethering ropes to ensure adhesion between EPEC and infected IEC. It also plays a role in inter-bacterial binding, helping EPEC adhere to each other, to form microcolonies. Microcolonies are formed by clusters of EPEC, often found in a cloud like formation on the surface of IEC. After EPEC’s initial attachment, a protein known as the translocated intimin receptor (Tir) is inserted into the host-cell membrane by EPEC’s type 3 secretion system (T3SS) [81]. The T3SS acts as a molecular syringe to translocate bacterial protein (known as effectors) into host cells. Tir is critical to the pathogenesis of EPEC and serves two unique functions. First, Tir acts as a receptor for the intimin protein, which is located on the outer membrane surface of EPEC [82,83]. This is an interesting example of a pathogen inserting its own receptor into the host cell membrane to aid its binding and attachment to host cells. On binding to the intimin protein, the intracellular region of Tir initiates a complex signalling cascade within the host cell to mediate actin rearrangements and pedestal formation [84,85]. Bacterial mutants lacking in Tir, intimin or the entire T3SS are severely attenuated in virulence. The T3SS as well as several key effector proteins are encoded within the LEE pathogenicity island [86]. Aside from Tir, the LEE region encodes five different T3SS-dependent effectors including
Mitochondrial-associated prote in (Map), EPEC secreted prote in (Esp) F, EspG, EspH and EspZ. Additional effector proteins have also been identified outside the LEE-encoded region. These non-LEE (Nle) encoded effector genes are located within six pathogenicity islets scattered throughout the EPEC and C. rodentium genomes [86,87]. The complete effector repertoire and their currently identified virulence functions have recently been reviewed elsewhere [26,27,88]. These virulence strategies involve pedestal formation, actin rearrangement, microtubule and epithelial barrier disruption as well as the modulation of inflammatory responses (summarized in Table 1). Although it has been shown that effector proteins such as EspZ counteracts host induce cell death to prevent epithelial shedding [89], EPEC also induced apoptosis through effector prote in EspF. This is thought to promote infection since induction of epithelial cell death is believed to aid the egress of the pathogen from the host [25]. EspF mutants failed to induce apoptosis in host cells [90,91]. Similarly, transfection of EspF into epithelial cells resulted in rapid host cell death that largely resembled apoptosis, as it disrupted mitochondrial membrane potential [90,91]. Aside from preventing host induced cell death, several groups have shown that mammalian epithelial cells infected with wildtype EPEC produce lower levels of inflammatory cytokines compared to cells infected by mutants lacking a functional T3SS. However, the exact mechanisms and the effector involved in the suppression are not yet completely known.
Table 1.1: EPEC effectors and their known functions

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Function</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Tir</td>
<td>Actin polymerisation, Tight junction disruption, Cell detachment, inflammatory suppression</td>
<td>[92,93,94]</td>
</tr>
<tr>
<td>Map</td>
<td>Tight junction disruption, microvilli effacement, mitochondria dysfunction</td>
<td>[93,95]</td>
</tr>
<tr>
<td>EspF</td>
<td>Disruption of tight junctions, induction of apoptosis, aquaporin redistribution, membrane remodeling</td>
<td>[96,97]</td>
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<tr>
<td>EspG</td>
<td>Microtubule disruption, tight junction disruption, induction of paracellular permeability</td>
<td>[98,99]</td>
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<tr>
<td>EspH</td>
<td>Cytoskeleton disruption, modulation of actin dynamics</td>
<td>[100]</td>
</tr>
<tr>
<td>EspZ</td>
<td>Promotes host cell survival</td>
<td>[89,101]</td>
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<tr>
<td>NleA</td>
<td>Inhibits protein secretion</td>
<td>[102,103]</td>
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<tr>
<td>NleB</td>
<td>Suppresses host inflammatory responses</td>
<td>[104,105,106]</td>
</tr>
<tr>
<td>NleE</td>
<td>Suppresses host inflammatory responses</td>
<td>[106,107]</td>
</tr>
<tr>
<td>NleH1,2</td>
<td>Suppresses host inflammatory responses</td>
<td>[108,109]</td>
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1.3.2.3 Mouse model of A/E pathogen infection: *Citrobacter rodentium*

Until recently, the lack of a small-animal model has largely restricted the study of EPEC pathogenesis to *in vitro* experiments, as EPEC does not effectively colonize/infect the intestines of mice or other laboratory animals [110]. However, our laboratory has been one of the leading groups using the murine pathogen *C. rodentium* as a model of EPEC and EHEC infection. A natural bacterial pathogen of mice, *C. rodentium* is a Gram negative bacterium related to EPEC and EHEC [111]. *C. rodentium*, formally called *Citrobacter freundii* biotype 480, is the only known LEE-positive organism pathogenic for rodent species [79]. In infected mice, *C. rodentium* infects epithelial cells in the colon and cecum, causing both mild diarrhea and colitis [112]. Infection by *C. rodentium* lasts approximately three weeks, and is characterized by acute colitis, as well as epithelial cell proliferation that leads to both crypt hyperplasia and goblet cell depletion. The infectious colitis caused by *C. rodentium* involves the localized recruitment of granulocytes, monocytes and lymphocytes [113]. Typically, *C. rodentium* causes a self-limiting infection leading to only modest morbidity and low rates of mortality in most mouse strains (C57BL/6, NIH Swiss and Balb/c) except C3H/HeJ and C3HOu/J mice, which are extremely susceptible to infection and suffer high mortality rates [114].

Following oral gavage, *C. rodentium* initially colonize the intestinal epithelium overlying the cecal patch (the lymphoid tissue in the cecum) of infected mice [112]. As the infection progresses, the pathogen spreads to the surrounding cecal epithelial cells over the first 2-3 days [113]. The infection progresses to the large intestine, colonizing the distal colon and rectum within 4-6 days after oral gavage. The peak of infection is generally around 8-10 days
post-infection (pi) but varies depending on the genetic background of the mouse as well as the composition of its microbiota [115,116]. Being a non-invasive pathogen, *C. rodentium* covers the mucosal surface, intimately adhering to the apical surface of colonic and cecal epithelial cells. Furthermore, at the peak of infection (days 8-10 pi), the adaptive immune response is activated [116]. The combination of IgG production and cytokine production by CD4+ T helper (Th)1 and Th17 cells ultimately clears the pathogen from infected animals, although the specific mechanisms of clearance remain unclear [117,118,119]. Infection begins to clear by day 14 pi, but mucosal thickening can be observed until day 21 pi. By day 28 pi the infection is typically resolved and the mice are refractory to re-infection [112].

As a non-invasive pathogen that resides entirely within the intestine, *C. rodentium* is an ideal organism to investigate A/E bacterial pathogenesis as well as the mucosal host responses generated against A/E pathogens [120]. Furthermore, since *C. rodentium* is effective at colonizing and infecting all laboratory mouse strains, it has helped our group and others identify the importance of host genetic background in controlling the outcome and extent of infection [40,110,111,112,113,114]. Similarly, the ability to use mice with gene specific knockouts as well as conditional knockouts with this model allows for the functional analysis of key genes during infection in a cell-type specific manner. On the pathogen side, as mentioned, *C. rodentium*, like EPEC and EHEC, contains a LEE pathogenicity island that enables this pathogen to cause A/E that are indistinguishable from those caused by EPEC [111]. Furthermore, *C. rodentium* contains all the LEE encoded and non-LEE encoded pathogenicity islands from EPEC and has repeatedly been shown to be a highly useful tool to investigate A/E bacterial virulence factors *in vivo* [87].
1.3.3 Commensal microbes and their impact on pathogen infection of the GI tract
As mentioned earlier, the presence of the commensal microbiota in the intestinal lumen is critical for the development and maturation of the mucosal immune system. Studies from germ-free animals have revealed that the commensal microbiota plays an important and active role in shaping the innate immune system [12,39]. Germ-free animals are defined as animals that are raised under sterile conditions, free of exposure to microorganisms. It is well established that animals born and raised in germ-free environments have stunted/dysregulated immune systems. This includes reduced numbers and sizes of Peyer’s patches and decreased numbers of intestinal immune cells such as plasma cells and T cells. Furthermore, the lack of commensal microbiota has been shown to impact tissue development in the cecae of germ-free animals, resulting in intestines that are thin walled and swollen, as compared to the intestines of animals raised under conventional conditions [7]. Therefore, the crosstalk between the innate immune system and the commensal microbiota is an important signal for the development of a proper immune system. As well, the commensal microbiota contributes to host responses in other ways. For instance, the commensal microbiota is thought to compete against enteric pathogens for space and essential nutrients at the mucosal surface [121,122]. The commensal microbiota therefore acts as a competitive barrier against colonization and invasion by enteric pathogens. This has been termed “microbial interference” or “colonization resistance” [68,123].

1.3.4 Colonization resistance
The health of an individual’s commensal microbiota can be determined by both its abundance, and its diversity, ideally filling a wide range of available environmental niches within the intestine [124]. In effect, this means that any invading pathogen will come into
direct competition with the indigenous commensal microbiota for both space and nutrients. Evidence from studies using antibiotics to deplete the commensal microbiota have demonstrated that enteric pathogens benefit from the resulting reduction in competition as this frees environmental niches and nutrients for the invading pathogens to exploit. For instance, risk of infection with the opportunistic pathogen, *Clostridium difficile*, is of a greater risk for colonizing and infecting the GI tracts of people who have undergone antibiotic treatment [125]. Similar observations were made in mice for *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) infection. In fact, the current gold standard for modelling *Salmonella* induced colitis in mice depends on the removal of commensal microbes by pre-treating mice with the antibiotic streptomycin [126,127]. It is not clear how in the absence of antibiotics, enteric pathogens are able to overcome the colonization resistance generated by most host’s commensal microbiota. However, it is clear that during infection, host responses to pathogens are capable of removing the commensal microbiota. For example, it was recently shown that infection of mice with *C. rodentium* leads to a drastic reduction in the total number of colonic microbiota, occurring in concert with the spread of the pathogen through the lower intestine [64]. Furthermore, the use of antibiotics during EHEC infection is contraindicated since it is believed that antibiotics causing the lysis of EHEC can cause large amounts of Shiga toxin to be released, potentially increasing the risk of hemolytic uremic syndrome [128]. Hence, the standard therapy for EHEC infection is rehydration therapy.
1.4 Innate host defense by IEC at the intestinal mucosal surface

Standing at the interface between the host and the luminal/external environment, the IEC barrier is under constant exposure from luminal antigens and microbes. On the one hand, IEC must try to sense and prevent infections by enteric pathogens, but on the other hand, IEC have to carry out their ion and nutrient transport functions, as well as maintain harmony with the commensal microbiota. As such the host immune system has developed to specifically tolerate the presence of commensal microbiota by reducing immune responsiveness at the mucosal surface (the IEC) yet still possess the ability to react rapidly to invading microorganisms. While the mechanisms allowing the immune system to distinguish between commensal microbes and enteric pathogens are unclear, innate receptors such as interleukin-1 receptor (IL-1R), Toll like receptors (TLR), and nucleotide-binding oligomerization domain (Nod) like receptors are likely to be involved, as they have been shown to play a critical role in intestinal host defense [16,19,129,130]. In fact, IEC can actively respond to pathogenic bacteria and modulate their responses in ways that promote microbial clearance. These strategies include the programmed death and shedding of infected IEC, increased production and release of antimicrobial peptides, and the modulation of the adaptive immune response. It has been shown that many if not all of these responses are dependent on MyD88 signalling and NF-κB activation [131,132]. In the studies outlined in this thesis, one of my primary goals was to examine the role of IL-1 receptor (IL-1R) family and toll-like receptor (TLR) signalling during enteric infection by A/E pathogens. These innate receptors, expressed by many cell types including IEC, contribute to host defence of the intestinal mucosa by recognizing molecular patterns present on pathogens, subsequently triggering the secretion of pro-inflammatory chemokines and cytokines, as well as antimicrobial factors.
1.4.1 The interleukin-1 receptor (IL-1R) superfamily

Both IL-1R family and TLRs are innate receptors that belong to the IL-1R superfamily [133,134]. All members of the IL-1R superfamily contain an intracellular Toll-IL-1 receptor (TIR) domain, which mediates a highly conserved signalling network promoting pro-inflammatory responses. The IL-1R superfamily can be further divided into two subfamilies based on their extracellular domains (Figure 1.4). The ILRs subfamily has immunoglobulin domains responsible for ligand binding. The other subfamily, the TLRs, have leucine-rich repeats (LRR) in their extracellular domain that can directly recognize conserved microbial components known as microbial associated molecular patterns (MAMPs). Both types of receptors play a critical role in initiating and shaping innate and, adaptive immune responses during infections.
Figure 1.4: The known members of TOLL-IL-1R superfamily and their ligands.

(A) The known members of TOLL-IL-1R superfamily and their ligands (B) Updated nomenclature and members of the IL-1 family. Figure modified from [133] and [135] with permission.
1.4.1.1 IL-1Rs subgroup

The IL-1Rs subgroup includes those receptors that bind to IL-1 related cytokines such as IL-1 (α and β), IL-18 and IL-33 [133]. Members of this subgroup include the Type I IL-1 receptor (IL-1RI), IL-1RII, IL-1 receptor accessory protein (IL-1RAcP), IL-18R, IL-18AcP (also termed AcPL), ST2, IL-1Rrp2, and the orphan receptors, IL-1 receptor associated protein-like (RAPL) and the negative regulator Single Ig-IL-1 related receptor (SIGIRR). IL-1RI and IL-1RAcP are the primary receptors for the pro-inflammatory cytokines IL-1α and IL-1β. IL-1β has the capacity to provoke inflammation in all tissues while producing a pronounced positive feedback for inflammatory signaling. IL-1β was first described as a general growth factor for T lymphocytes and recently has been shown to be a key regulator of Th17 T cells [136]. IL-18R and IL-18AcP are the receptors for IL-18 and are expressed on T cells as well as NK cells, macrophages, B cells, neutrophils, basophils, and mast cells. The IL-18R is also expressed on non-haematopoietic cells such as endothelial cells, smooth muscle cells and epithelial cells [137,138,139,140]. IL-18 is an important co-stimulatory factor for interferon gamma (IFN-γ) production, thus helping to drive the development of Th1 T cells. In the intestine, IL-18 production was localized to IEC, macrophages or dendritic cells [141,142]. However, studies examining the role of IL-18 in colitis have been contradictory. On one hand, blocking IL-18 was found to ameliorate the severity of dextran sodium sulfate (DSS) induced colitis by limiting the infiltration of immune cells into the lamina propria [143]. On the other hand, recent studies demonstrated inflammasomes dependent production of IL-18 by IEC is important in maintaining mucosal integrity, epithelial proliferation and in preventing translocation of bacteria across the intestinal epithelial barrier [144,145]. Similarly, IL-18 can induce neutrophil transmigration across IEC without altering epithelial permeability in vitro [146] supporting a role for IL-18 in
maintaining epithelial integrity. However, despite its prominent role in promoting mucosal homeostasis during DSS colitis, IL-18 was not found to play a critical role during C. rodentium infection [147].

**1.4.1.2 The TLR subgroup**

During the past two decades, TLRs have received significant attention as these germline-encoded pattern recognition receptors (PRRs) were found to recognize highly conserved molecular structures in bacteria called PAMPs [148,149]. There have been 10 TLRs identified in humans and 12 have been identified in mice with each TLR recognizing different lipids, lipoproteins, proteins or nucleic acids (Figure 1.4), derived from a wide range of bacteria, viruses, parasites or fungi. For this thesis, I will focus on describing the TLRs that recognize enteric bacterial pathogens.

TLR4 recognizes lipopolysaccharides (LPS), which make up part of the outer membrane of Gram-negative bacteria [150,151]. Within host cells such as macrophages, TLR4 forms a complex with MD2 on the cell surface, and together they serve as the main LPS-binding component. In addition to MD2, LPS binding protein (LBP) and a molecule called the cluster of differentiation (CD) 14 also assist in the binding of LPS. LBP is a soluble plasma protein that binds LPS while CD14 acts as a co-receptor to the TLR4-MD2 complex [151]. Another membrane surface TLR is TLR2, which recognizes many bacterial (peptidoglycan), fungal (zymosan) and viral (hemagglutinin protein from measles virus) ligands. TLR2 is able to recognize a diverse array of ligands through its ability to form heterodimers with either TLR1 or TLR6. The TLR2-TLR1 heterodimer recognizes triacylated lipoproteins from Gram-negative bacteria [152], while the TLR2-TLR6 heterodimer recognizes diacylated
lipoprotein from Gram-positive bacteria [153]. TLR5 recognizes the flagellin component of bacterial flagella [150]. There are highly conserved regions within the flagellin protein (produced by flagellated bacteria), that facilitates the recognition of flagellin by TLR5. TLR9 recognizes unmethylated cytidine-phosphate-guanosine (CpG) deoxyribose nucleic acid (DNA) sequences. Unmethylated CpG motifs are common in bacterial and viral DNA but extremely rare in the DNA of mammalian cells. TLR9 stimulation is known to drive strong Th1 responses against microbial infection [154].

1.4.1.3 The MyD88 dependent pathway for IL-1Rs/TLRs signalling

Following activation by their respective ligands, IL-1R/TLR proteins homodimerize or heterodimerize and initiate signaling cascades by recruiting adaptor molecules for further downstream activation [133,149,155,156,157,158]. All IL-1R/TLR, except for TLR3, use myeloid differentiation primary response gene 88 (MyD88) as an adaptor protein to initiate their signalling. In some cases, such as the activation of TLR4, the TLR1-TLR2 heterodimer and the TLR2-TLR6 heterodimer, MyD88 acts in concert with other adaptor molecules such as the “MyD88 adapter like” (MAL) protein to initiate inflammatory signaling cascades [132]. Once activated, MyD88 recruits downstream kinases to aid in signaling. MyD88 recruits IL-1R associated kinases (IRAK) 4 and IRAK1 to form macromolecular complexes, which lead to the activation of tumour necrosis factor related associated factor 6(TRAF6). TRAF6, in turn, recruits transforming growth factor β activated kinase 1 (TAK-1), leading to activation of the NEMO complex which results in the degradation of IκB and the release of the transcription factor nuclear factor kappa B (NF-κB) p65/Rel A subunit. The released NF-κB translocates from the cytosol into the nucleus, where it then activates NF-κB dependent
genes. Aside from releasing NF-κB from the IKK complex, TAK1 also activates mitogen activated protein kinases (MAPs) such as Erk1, Erk2, p38 and c Jun N-terminal kinases (Jnk) to induce other transcription factors such as activator protein (AP)-1, resulting in the production of inflammatory cytokines (Figure 1.5) [155,156,157,158].

Aside from MyD88, TLR4 signaling can also employ another adaptor molecule called TIR domain containing adapter inducing interferon β (TRIF) [159]. Interestingly, although TLR4 appears to be able to signal through both MyD88 dependent and TRIF dependent pathways, activation of these two pathways is mutually exclusive. The TRIF dependent pathway inhibits activation of the MyD88 dependent pathway by degrading MyD88 following TLR4 stimulation [160]. Overall, activation of IL-1R/TLR signalling through either MyD88 dependent or independent pathways results in the translocation of transcriptional factors such as NF-κB, AP-1 and IRF-3 into the nucleus. These transcriptional factors initiate inflammatory responses such as production of antimicrobial peptides and inflammatory cytokines to eradicate invading microbes and initiate tissue repair [18,19,22].
Figure 1.5: MyD88 dependent and independent signalling pathways

TLR and ILR stimulation activates either MyD88 dependent or MyD88 independent signaling pathways. The intracellular signalling cascades lead to translocation of NF-κB, AP-1 and IRF-3 into the nucleus.

1.4.1.4 Spatial distribution and expression of TLR within IEC

IEC are known to express all of the IL-1Rs, and they all appear functional [22,161]. The function of TLRs within IEC is less clear. Almost all TLR are expressed at the mRNA level in IEC but there are differences in their expression levels and distribution within these cells. In general, TLR expression by IEC is low and many receptors are expressed only on the basolateral side of IEC thereby limiting their potential interactions with bacterial PAMPs within the gut lumen [161] (Figure 1.6). For instance, it has been shown that TLR2 and TLR4 are expressed at low levels by IEC in healthy human colons [162] and are mainly
expressed within endosomal compartments under homeostatic conditions. Furthermore, to prevent unnecessary activation by LPS, MD-2 and CD14, co-receptors for TLR4, are expressed at only a low level under homeostatic conditions [162,163]. TLR5 expression by IEC predominates in the colon but TLR5 expression is restricted to the basolateral side of polarized epithelial cells, presumably to prevent excessive stimulation from luminal flagellin [161]. TLR9 is found on both the apical and the basolateral membranes of IEC, but ligand binding at these sites initiates different cellular signalling [164,165]. TLR9 stimulation at the apical plasma membrane dampens NF-κB signalling within the epithelium and thus supports mucosal tolerance towards microbial exposure. In contrast, stimulation of TLR9 on the basolateral surface strongly stimulates proinflammatory chemokine secretion through NF-κB activation [164,165].
IEC express all TLRs; however their spatial distribution is modified to limit activation by commensal microbes. Picture reproduced from [161] with permission)

1.4.2 Active role of IEC in host defense: secretion of anti-microbial peptides

Additional actions of IEC in innate host defense include their production and apical release of anti-microbial peptides that are thought to produce a bacteriostatic/bacteriocidal zone above the IEC, helping to prevent bacteria from reaching and/or penetrating the gut epithelium during infection [9,23,40,51]. Enterocytes in the colon produces several antimicrobial factors including β-defensins, regenerating islet-derived protein 3 gamma (Reg 3γ) and cathelicidin [166,167]. Human β-defensin 1 is expressed constitutively by enterocytes while the expression levels of human β-defensin 2 and human β-defensin 3 are upregulated following exposure of IEC to microbial products and inflammatory cytokines. The expression of these and other anti-microbial factors by the colonic epithelium and mucosa is known to be influenced by TLR and MyD88 dependent signaling. For example, Reg 3γ is secreted by IEC and promotes host defense against the Gram-positive bacterial
pathogen *Listeria monocytogenes* in a MyD88 dependent manner [168]. Moreover, attenuation of NF-κB signaling specifically within the colonic epithelial cells of mice through conditional ablation of IKKγ or NEMO, was found to lead to decreased production of the antimicrobial β-defensin 2, which was found to correlate with increased resident bacterial translocation into mucosal tissues under colitic conditions [169]. Hence, IEC reinforce their structural barrier function by abundantly producing and secreting anti-microbial products including anti-microbial peptides, into the overlying mucus layer or into the intestinal lumen, thereby forming a distinct physiochemical defense barrier.

### 1.4.2.1 Active role of IEC in host defense: epithelial proliferation and shedding

Aside from the increased secretion of anti-microbial peptides in response to infection, increased IEC proliferation and/or increased IEC apoptosis or sloughing into the intestinal lumen are also often observed during infections [24,25,40]. These responses appear to reflect a defense mechanism involving the replacement of infected cells with newly arisen and hence uninfected IEC. IEC undergo cell death and cell shedding which contributes to epithelial turnover under physiological conditions. During infection, damaged IEC are rapidly exfoliated from the epithelium along with any pathogens that have infected those specific IEC, thereby aiding in the clearance of infections [170]. As the rate of epithelial cell death or shedding increases, crypts have to increase stem cell proliferation to replace the exfoliated cells and to maintain epithelial integrity. In many cases, increased IEC proliferation becomes greater than what is required to replace sloughed IEC, resulting in a lengthening of colonic crypts, referred to as hyperplasia [40]. There is significant evidence that MyD88 signalling and NF-κB activation regulate cellular proliferation as well as
apoptosis [171]. For example, TLR2-bacterial lipoprotein interactions can activate the extrinsic pathway of apoptosis via signalling involving MyD88 [172]. Moreover, TLR4-LPS interactions have been demonstrated to induce apoptotic pathways in macrophages under conditions of proteasomal blockade [173]. Epithelial deficiency in the NF-κB subunit p65 or IKK leads to increased apoptosis of IEC, loss of barrier integrity and increased translocation of commensal bacterial in the colon following challenge with DSS [171]. Therefore, NF-κB and TLR signalling are important contributors to epithelial proliferative and apoptotic responses within the GI tract, in order to maintain barrier integrity. In the event that the IEC barrier is breached by a bacterial pathogen, IEC are also capable of recruiting profession immune cells to assist in the removal of pathogens by releasing chemokines and cytokines.

1.4.2.2 Active role of IEC in host defense: modulation of immune function

Another important role of IEC in active host defense is through the modulation of the immune system. From cell culture experiments using colonic epithelial cell lines, it is evident that following IL-1β stimulation, IEC rapidly releases chemokines and cytokines in a p38 MAP kinase and NF-kB dependent manner [174,175]. These chemokines include CXCL8 (formerly known as IL-8), monocyte-chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2). CXCL8 is a potent chemoattractant and activator of leukocytes while MCP-1 and MIP-2 are chemokines that play a major role in intestinal inflammation by recruiting monocytes and neutrophils [176,177]. Aside from modulating innate inflammatory cells in the lamina propria by releasing chemokines and cytokines, recent studies have demonstrated that IEC also shape the adaptive immune response by modulating T cell activation and participating in antigen presentation. Thymic stromal
lymphopoietin (TSLP) is a cytokine that activates dendritic cells involved in the positive selection of regulatory T cells. Regulatory T cells are a component of the immune system that suppresses the immune responses of other cell types. TSLP is expressed constitutively by IEC and its expression can be enhanced in response to infection, inflammation and tissue injury in an NF-κB-dependent manner [178]. IEC also express receptors that can function in antigen presentation, but their specific role during enteric bacterial infections is unclear.

1.4.3 Regulation of Toll-IL-1R signaling within IEC

Remarkably, despite IEC expression of innate receptors such as TLRs, they are generally considered hypo-responsive to most bacterial products, at least under normal physiological conditions. Though tissue specific deletion of MyD88 in IEC, it was found that MyD88 in IEC plays a limited role in protecting mice from DSS induced colitis. Instead, it was shown that MyD88 signaling from B cells protected mice from DSS challenge [179]. The host uses both passive and active strategies to regulate TLR activation in IEC. These strategies include decreasing the expression of TLRs, and compartmentalizing the expression of TLRs and their co-receptors to dampen NF-κB activation. Aside from these passive strategies, IEC also use negative regulators of innate signaling to down-regulate innate signaling [18,20,21].

1.4.3.1 Negative regulators of IL-1R/TLR signalling

Negative regulators limit IL-1R/TLR signalling by interfering with, or dampening the activation cascade in epithelial cells (Figure 1.7). Peroxisome proliferator-activated receptor γ (PPAR-γ) and the cytoplasmic zinc finger protein A-20 interfere with NF-κB activation by flagellin stimulation [180,181]. Initially, PPAR-γ was found to prevent translocation of NF-
κB into the nucleus. More recently, commensal microbes have been described to upregulate
PPAR-γ expression within IEC, thereby attenuating NF-κB activation in a TLR4 dependent
manner [182]. A-20 is the only negative regulator to date that regulates both MyD88
dependent and MyD88 independent pathways in epithelial cells [183]. A-20 is rapidly
induced after LPS stimulation in macrophages and prevents TRAF6 activation, thereby
attenuating NF-κB activation.

Figure 1.7: Schematic diagram showing the factors that negatively regulate TLR signaling.
IEC utilize negative regulators such as SIGIRR, TOLLIP and A-20 to negatively regulate TLR/IL-1R
signalling.

As mentioned previously, the IRAK1-IRAK4-TRAF 6 complex is critical in IL-1R/TLR
signaling cascades. Not surprisingly, they are also targets for negative regulation or inhibition.
of IL-1R/TLR signalling. Unlike other IRAK molecules, which are ubiquitously expressed, IRAK-M expression is limited to myeloid cells. Similar to A-20 where its expression increases following LPS stimulation, IRAK-M prevents NF-κB activation by interfering with the dissociation of the IRAK-1-IRAK-4 complex [184]. IRAK-M-deficient mice challenged with S. Typhimurium exhibit an increased number of grossly enlarged Peyer's patches suggesting the development of enhanced innate immune response against the infection. Another inhibitor molecule that functions by interfering with the IRAK complex is Toll interacting protein (Tollip), which is an inhibitor for IL-1 and NF-κB activation (Figure 1.7). Following exposure to TLR ligands, such as LPS, IEC upregulate Tollip expression thereby limiting their pro-inflammatory response to a secondary exposure to TLR ligands [185]. Aside from these intracellular negative regulators, the decoy receptor SIGIRR also negatively regulates IL-1/TLR signaling [186]. Expressed primarily by epithelial cells, SIGIRR has been shown to play a critical role in regulating IEC innate responses in order to maintain intestinal homeostasis.
1.4.4 Single Ig-IL-1 related receptor (SIGIRR)

Belonging to the IL-1R superfamily, SIGIRR, also known as TIR8, is a negative regulator of IL-1R and TLR signaling [186,187]. Unlike other members of the IL-1R family, SIGIRR only has a single Ig domain [188]. The extracellular domain of SIGIRR has been shown to interfere with the heterodimerization of IL-1RI and IL-1RAcP, thus interfering with IL-1R signaling [189]. Although it contains an intracellular TIR domain, SIGIRR lacks the necessary components to initiate downstream signaling [186]. Instead, the TIR domain inhibits IL-1R/TLR signalling by sequestering the adaptor molecules IRAK and TRAF-6 to dampen NF-κB activation (Figure 1.7.) While first described as a negative regulator of TLR4 and IL-1R signalling, SIGIRR also suppresses pro-inflammatory signalling from other TLRs and IL-1Rs that utilize MyD88 dependent pathways. The immune suppression is specific as SIGIRR inhibits IL-1R and TLR induced NF-κB and JNK activation but not TNF-α dependent NF-κB activation or IFN-γ dependent STAT-1 activation. Although its immune suppressive role has been well documented, the regulation of SIGIRR expression remains poorly defined. LPS and inflammation generally lead to a down-regulation of SIGIRR gene expression but, on the other hand, Th2 polarization of T cells and exposure to vasoactive intestinal peptide (VIP) in cornea cells has been shown to increase expression of SIGIRR [190,191,192,193].

SIGIRR is ubiquitously expressed in all tissues but its expression level differs between cell types [186]. Expressed primarily by epithelial cells and T lymphocytes, it is also detectable in monocytes and dendritic cells. While SIGIRR is best known for limiting inflammatory responses induced by LPS and IL-1, SIGIRR has also been shown to suppress mammalian
target of rapamycin (mTOR) dependent Th17 cell differentiation [194]. Based on its expression profile and subsequent experimental examinations, SIGIRR has been shown to play a critical role in limiting autoimmune and inflammatory diseases, including limiting the severity of DSS induced colitis in mice [195,196,197]. The roles played by SIGIRR in regulating inflammation at systemic sites was recently reviewed by Riva et al. [198]. I will therefore focus on outlining the current knowledge regarding the roles played by SIGIRR within the intestine.

1.4.4.1 Role of SIGIRR in IEC

SIGIRR is expressed primarily by mammalian epithelial cells, including IEC; therefore much work has been done to examine its effects at mucosal surfaces. Not surprisingly, based on its broad impact on innate signalling, SIGIRR has been shown to play a critical role in maintaining mucosal homeostasis [195,199]. For instance, SIGIRR deficient (-/-) mice develop a low-level but constitutive NF-κB and JNK activation within their IEC [195]. As a result, the gene expression of several pro-inflammatory chemokines and cytokines are significantly augmented in IEC under homeostatic conditions. Furthermore, the expression of SIGIRR by IEC is known to decrease in inflamed intestinal tissues, such as those from ulcerative colitis patients, and from mice during DSS colitis [193]. These findings suggest SIGIRR expression in IEC can be modulated under inflammatory conditions. Indeed, studies by our laboratory found that bacterial infection of IEC as well as their exposure to flagellin transiently decreased SIGIRR protein expression [192]. Although the mechanism of regulation of SIGIRR expression remains elusive, it is clear that SIGIRR attenuates TLR and IL-1R stimulation. Our laboratory demonstrated stable overexpression of SIGIRR diminished NF-κB–mediated CXCL8 secretions to TLR ligands and IL-1β. In contrast, suppression of
SIGIRR by small interfering ribonucleic acid (siRNA) significantly enhanced colonic epithelial cell line (Caco-2 and HT-29 cells) (production of IL-8) responses to these ligands [192]. Indeed, SIGIRR interactions with microbes through TLR signalling are important in regulating mucosal homeostasis. Our group recently helped to show that SIGIRR plays a crucial role in regulating homeostasis, inflammation and tumorigenesis in the mouse colon. *Sigirr*−/− mice suffered extensive mucosal damage and increased mortality following DSS induced colitis [195]. The protective role of SIGIRR in this model was shown to reflect exaggerated survival signalling within IEC that was driven by the presence of commensal microbiota. While SIGIRR has been shown to play a critical role in intestinal homeostasis, its impact on host responses to enteric bacterial infections has not yet been examined.

### 1.4.5 Innate responses to *Citrobacter rodentium* infection

Our laboratory has shown that MyD88 dependent signaling is important to protect against *Citrobacter rodentium* infection. Mice lacking MyD88 are unable to control *C. rodentium* growth, and suffer severe necrosis of their colonic tissues, indicating that MyD88 signalling also limits tissue damage during infection by this pathogen [200,201]. Our laboratory also noted MyD88-dependent defects in the production of inducible nitric oxide synthase (iNOS) which also contributes to direct killing of this pathogen [202]. There are also major defects in epithelial proliferative responses in *Myd88*−/− mice, as infection induced robust epithelial proliferation and crypt hyperplasia in wildtype mice, but not in *Myd88*−/− mice [200]. *C. rodentium* activates both TLR2 [203] and TLR4 [204]. Mice deficient in TLR4, while highly susceptible to DSS induced colitis [205], displayed a surprising delay in *C. rodentium* colonization [204] compared to wildtype mice. This was characterized by a marked reduction
in *C. rodentium* burdens in the first week of infection. Even when *C. rodentium* did eventually colonize to wildtype levels, the host inflammatory response was impaired, as *Tlr4* -/- mice displayed reduced expression of the chemokines MCP-1 and MIP2α, along with limited tissue infiltration of neutrophils and macrophages. Ultimately, *C. rodentium* was able to colonize and cause inflammation in *Tlr4* -/- mice, but the mice suffered modest tissue damage and were still able to clear the infection. In contrast, *Tlr2* -/- mice were highly susceptible to *C. rodentium* infection, exhibiting rapid weight loss and high mortality levels between 1 and 2 weeks post-infection. TLR2 deficiency led to severe ulceration in the mid and distal colon. Surprisingly, this was not due to higher *C. rodentium* burdens, as colonization was similar to wildtype mice [203], but instead involved reduced production of the cytokine IL-6, leading to exaggerated epithelial cell apoptosis during infection.

While MyD88 signalling is a central player in host defense against *C. rodentium*, *Myd88* -/- mice are impaired not only in the signalling of TLRs, but also of the cytokines IL-1β and IL-18. Following *C. rodentium* infection, mice lacking IL-18 suffered only modest defects in host defense, whereas mice lacking the IL-1R were more susceptible to *C. rodentium*, developing exaggerated mucosal damage, albeit later and to a lesser degree than that observed in *Myd88* -/- mice. The *Il-1r* -/- mice did not harbor higher pathogen burdens, or defects in epithelial proliferation or neutrophil recruitment [147], suggesting that these aspects of the phenotype seen in infected *Myd88* -/- mice likely reflect the loss of TLR signalling.
1.5 Research objectives:

I have thus outlined that within the mammalian colon, innate signaling plays an important role in promoting both inflammation as well as epithelial homeostasis in response to infection by bacterial pathogens, such as *C. rodentium*. Despite the fact that many of the responses to infections reflect changes in IEC function, it is unclear whether protective innate signaling occurs within the IEC, or in other cell types within the GI tract. We know that IEC are generally, innately hypo-responsive to most TLR and IL-1R ligands, and this hypo-responsiveness likely helps prevent spontaneous inflammation from developing in response to the presence of the intestinal commensal microbiota. It is unclear, however, whether this hypo-responsiveness actually impairs effective host responses against enteric bacterial pathogens, since presumably a more innate reactive epithelial layer should be able to respond more effectively to invading pathogens.

Our previous studies have identified Sigirr as playing a critical role in suppressing innate IEC responses to innate ligands as well as intact bacterial pathogens like EPEC [192]. Through the use of the *C. rodentium* infection model, I will examine the role of the mammalian host protein SIGIRR during *C. rodentium* infection. In this thesis, my first goal will be to explore the impact of SIGIRR in controlling host responses to *C. rodentium* infection, clarifying whether loss of SIGIRR during *C. rodentium* infection, impacts host commensal microbiota. At present, very little is known about what factors control pathogen colonization resistance within the mammalian GI tract, however recent studies have found that the host inflammatory response triggered by *C. rodentium* and other pathogens can actually facilitate pathogen colonization of the intestinal lumen and mucosa [64]. While the mechanisms are
unclear, inflammation may alter the intestinal luminal environment in many ways, including the removal of competing commensal bacteria and the disruption of normal homeostasis. The *Sigirr* -/- mice exhibit a higher inflammatory tone without developing spontaneous diseases; therefore, these mice may offer an excellent tool to identify the host factors that facilitate pathogen colonization of the GI tract. These studies may also clarify whether the main function of SIGIRR is to dampen inflammatory (or other) responses that may be subverted by bacterial pathogens to promote their colonization. Excessive inflammatory responses have been suggested to remove the resident commensal microbiota lowering competition against enteric pathogens.

Second, it is clear that pathogens use a variety of means to overcome host defence, in order to successfully infect their hosts. One of the strategies used by enteric bacteria such as EPEC and EHEC is the hijacking of host cellular processes by bacterial effectors [175,206]. My second goal in this thesis is to explore how these and related enteric pathogens modulate intestinal inflammatory responses to their advantage during infection. Like other Gram-negative enteric bacterial pathogens, EPEC and *C. rodentium* express virulence factors that promote colonization and infection of the intestinal mucosa. I wish to examine how EPEC and *C. rodentium* overcome the innate immune responses during infection by unmasking which effectors are involved. While it has been shown that EPEC suppresses inflammatory responses following *in vitro* infection, the effectors involved have not yet been identified. My goal is to identify the A/E bacterial effectors and mechanisms involved in the suppression of the host immune response. By utilizing the *C. rodentium* model, I plan to examine the role of bacterial effectors during disease progression *in vivo.*
Taken together, my thesis will thus clarify how IEC innate responsiveness and function are regulated, by both the host and by invading pathogens. My hope is that after reading my thesis, the central role played by the epithelium in providing host defense, and optimal GI health will become apparent. Moreover I hope that my studies will help explain how the mutualistic relationship between host and microbiota has developed to provide optimal host defense within the GI tract.
Chapter: SIGIRR limits colitic and epithelial homeostatic responses, but promotes microbiota dependent colonization resistance to enteric bacterial pathogens

2.1 Introduction

*Citrobacter rodentium* is a mouse-specific A/E bacterial pathogen related to the clinically important EPEC and EHEC. *C. rodentium* has been widely used to define the *in vivo* virulence strategies employed by A/E pathogens [40,111]. It has also proven a popular model to assess host immune responses against mucosal pathogens as well as explore how pathogens compete with intestinal commensal microbes for colonization niches and nutrients [64,207]. Our laboratory has shown that MyD88 dependent innate receptors play a critical role in driving the host response to infection as well as controlling *C. rodentium* burdens carried by mice. Moreover, MyD88 signalling plays an important role in protecting mucosal integrity by promoting IEC proliferation, barrier function and repair during infection [200,201]. While the full array of receptors involved in the protective actions of MyD88 is still unclear, TLR4 appears to drive the inflammatory response and IEC proliferation, while TLR2 and IL-1R both protect IEC integrity and drive IEC repair [40,147,204,208].

Since many of the MyD88 dependent responses to *C. rodentium* infection involve changes in IEC function or proliferation, I questioned whether they reflect MyD88 dependent signalling within the IEC themselves. While it is clear that MyD88 signalling can occur within IEC, in general, IEC are hypo-responsive to most bacterial products and other aspects of inflammatory signalling [209]. Since IEC exist in close proximity to trillions of commensal bacteria, their hypo-responsiveness may be necessary to maintain a mutualistic relationship
with the resident microbiota and prevent spontaneous intestinal inflammation [40]. The commensal microbiota, aside from providing nutrients to the host, are important contributors to host defense by promoting resistance to colonization by enteric pathogens. Disruption of the commensal microbiota (through antibiotic treatment, for example) enhances enteric pathogen colonization and subsequent disease in mice [68,127,210]. However, the molecular mechanisms that underlie commensal microbe based resistance to colonization in the mammalian intestine remain poorly defined.

Recent studies suggest that the innate hypo-responsiveness of IEC may in part reflect the actions of SIGIRR (also called TIR8), a negative regulator of IL-1R and TLR signaling. SIGIRR suppresses MyD88 dependent signaling by sequestering IRAK1 and TRAF6, both downstream targets of MyD88 [186,198,211]. A former doctoral student in our laboratory, Dr. Mohammed Khan recently showed over-expression of SIGIRR in IEC significantly dampened their secretion of inflammatory cytokines. In contrast, siRNA knockdown of SIGIRR dramatically increased inflammatory cytokine release by IEC in response to different TLR ligands [192]. In collaboration with Dr. Xiaoxia Li’s group, we have also shown that SIGIRR deficiency leads to exaggerated DSS induced colitis, that depended on the presence of commensal microbes and the loss of SIGIRR expression by IEC. However, the specific receptors regulated by SIGIRR were not defined [195]. To address what impact increased innate responsiveness by IEC would have on intestinal host defense, I tested the effect of SIGIRR on the host response to *C. rodentium* infection.
As previously reported, the intestines of uninfected *Sigirr* deficient (-/-) mice were histologically similar to those of WT mice [195,212] but following *C. rodentium* infection, *Sigirr* -/- mice developed exaggerated inflammatory and antimicrobial responses, along with increased mucosal damage and IEC proliferation. Bone marrow (BM) transplantation confirmed that loss of SIGIRR in the non-BM compartment (putative IEC) controlled the exaggerated colitic and IEC responses. These responses appeared largely independent of TLR2 or TLR4 but were instead dependent on IL-1R signalling. Despite their exaggerated colitic and antimicrobial responses, *Sigirr* -/- mice showed dramatically increased susceptibility to *C. rodentium* infection, in association with the rapid depletion of their intestinal commensal microbes. This loss of commensals reduced the host’s resistance to colonization by *C. rodentium* as well as to other enteric bacterial pathogens. Thus SIGIRR plays an unexpected role in enteric host defense, promoting commensal microbe-based colonization resistance to pathogens at the expense of limiting antimicrobial, colitic and IEC homeostatic responses.
2.2 Experimental procedures

2.2.1 Mouse strains and infection of mice

Mouse strains used in this study: C57BL/6 (WT), Sigirr -/-, Il-1r/Sigirr -/-, Tlr2/Sigirr -/-, Tlr4/Sigirr -/- and Myd88/Sigirr -/- mice (8-12 weeks) were bred in house, and kept under specific pathogen free conditions at the Child and Family Research Institute. All mouse strains used in this study are on C57BL/6 background. Il-1r1 -/- and Villin cre mice were purchased from Jackson Laboratory. MyD88 flox mice were from Dr. Xiaoxia Li’s laboratory. All double gene deficient mice were generated by crossbreeding single gene deficient mice with the genotypes of the resulting pups following a Mendelian ratio. IEC-MyD88 -/- mice were generated by crossbreeding MyD88 flox mice with Villin cre mice. Mice were orally gavaged with an overnight culture of ~2.5 x 10^8 CFU of streptomycin resistant C. rodentium DBS100 or 1 x 10^6 CFU of ΔaroA S. Typhimurium SL1344 and euthanized at specified time points pi. Infections were performed under both co-housing conditions (WT and mutant mice in same cage) and single housing conditions (WT and mutant in different cages). Mice were monitored for mortality and morbidity throughout infection and euthanized if they showed signs of extreme distress or >15% body weight loss. Body weight data are presented as the mean percentage of starting weight. All experiments were performed according to protocols approved by the University of British Columbia’s Animal Care Committee and in direct accordance with CCAC guidelines.

2.2.2 Tissue collection, bacterial counts and pathology scoring

Tissue collection and bacterial counts were performed as described previously [213]. Briefly, mice were anesthetized with isofluorane and euthanized over the course of infection, dissected and divided into cecum and colon to be collected in 10% neutral buffered formalin
(Fisher) for histological analyses, or processed for tissue pathology assays. For viable cell
counts, cecum, colon tissues and stool pellets were collected separately, and homogenized in
PBS pH 7.4, with dilutions plated onto LB agar or streptomycin agar plates. Pathology was
scored using a previously adapted scoring system [48]. In brief, paraffin-embedded colonic
tissue sections (5 µm) that had been stained with haematoxylin and eosin were examined by
two blinded observers. Tissue sections were assessed for i) submucosal edema (0- no change
1- mild 2- moderate 3- profound), ii) hyperplasia (0- no change 1-1-50% 2- 51%-100% 3 -
> 100%), iii) goblet cell depletion (0- no change 1- mild depletion 2- severe depletion 3-
absence of goblet cells), iv) epithelial damage/ integrity (0- no change 1- few cells slough,
epithelial surface rippled 2- epithelial surface is rippled, damaged 3- epithelial surface is
severely disrupted/damage, large amount of cells sloughing). For ulceration, an additional 1
was added for each 25% (or fraction of) of the tissue in the cross section affected (ie. a small
ulcer would score as 4+1, whereas a tissue where 60% of the tissue cross section is ulcerated
would be 4 + 3) v) mononuclear cells infiltration (per 40 x field) (0- no change 1- <20 2- 20
to 50 3- > 50 cells. The maximum possible score is 18.

2.2.3 RNA extraction and quantitative real-time PCR
Following euthanization of mice, tissues were immediately transferred to RNA-later
(Qiagen). Total RNA was purified using QiagenRNEasy kits (Qiagen) stored according to
manufacturer's instructions. cDNA was synthesized with Omniscript RT kit (Qiagen) and
OligodT (Applied Biological Materials Inc) followed by quantitative real-time PCR
techniques. qPCR was carried out on MJ Mini-Opticon Real-Time PCR System (Bio-Rad)
using IQ SYBR Green Supermix (Bio-Rad) and MIP-2 (, MIP3-α, β-actin, mCRAMP, Reg3-
γ, β-defensin III primers using sequences and conditions previously described [48].
Quantification used the Gene Ex Macro OM 3.0 software (Bio-Rad) where PCR efficiencies for each primer set were incorporated into the final calculations.

2.2.4 Immunostaining
Immunofluorescence staining of control and infected tissues was performed using previously described procedures [213]. In brief, paraffin embedded tissues were cut (6 µm) and stained for Ki-67 (Thermo) or phospho STAT-3 (Abm-Cam). Tissues were mounted using ProLong Gold Antifade reagent (Invitrogen) containing DAPI for DNA staining. Sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software version 4.0.

2.2.5 Cecal loop surgery
Cecal loop infections were performed as recently described [213]. In brief, mice were anaesthetized (isofluorane) and following a midline abdominal incision, the cecum was exposed. The proximal colon close to the cecum was ligated, thus isolating the cecum. ~1 x 10^7 CFU of *C. rodentium* were injected into the cecal loop which was returned to the abdominal cavity and the incision closed with discontinuous sutures. The mice were euthanized at 10 hr pi and tissues and stool contents were collected for CFU counts and mRNA analysis as described above (section 2.2.3).

2.2.6 Bone marrow reconstitution
Bone marrow was isolated from C57BL/6.Ly5.1 (WT), or Sigirr -/- mice (Ly5.2). Four million cells were injected intravenously into lethally irradiated wildtype (WT), or Sigirr -/- recipient mice that were allowed to recover for 12 weeks. Reconstitution was confirmed by
FACs analysis of peripheral blood leukocytes for Ly5.1 expression. Only mice exhibiting greater than 90% chimerism were infected. Similar methods were performed to obtain Sigirr -/- +WT BM mice.

2.2.7 Assessment of total microbes and FISH hybridization
Enumerating total microbes and FISH staining were performed as described previously [48]. At least 2 fecal pellets were collected from each animal at time points indicated. After homogenization, samples were placed in 10% Neutral Buffered Formalin to a final concentration of 3%. Samples were further diluted 1:10 in PBS, vortexed briefly, and stored at 4°C. 2–5 µl of the 1:10 diluted sample stored in PBS was diluted in 1 ml PBS and filtered onto Anodisc 25 filters (Whatman International Ltd) with a pore size of 0.2 µM and 2.5 cm diameter. The samples were allowed to thoroughly dry, and were then mounted on glass slides with ProLong Gold Antifade reagent with DAPI (Molecular Probes) and viewed as above (section 2.2.4). The mean number of cells counted in 3 to 6 randomly chosen fields per disc was determined by two scorers. For FISH staining, samples were incubated overnight at 37°C in the dark with Texas red-conjugated EUB338 general bacterial probe (5′-GCT GCC TCC CGT AGG AGT-3′) and an AlexaFluor 488 conjugated GAM42a probe (5′-GCC TTC CCA CAT CGT TT-3′) that recognizes bacteria that belong to the γ-Proteobacter class.

2.2.8 Colonic crypt killing assay
The bactericidal capacity of cecal crypt secretions was performed as described previously [214]. Cecal and Colonic crypts were isolated from the naïve WT and Sigirr -/- mice, resuspended in iPIPES buffer (10 mM PIPES; pH 7.4 and 137 mM Sodium Chloride), and counted. For experiments, 1000 crypts were incubated in 40 µL of iPIPES buffer at 37°C for
30 minutes. Following this period, 10 μL of a crypt-free isolate was added to $10^4 E. coli$ (commensal isolates, a gift from Dr. Ben Willing) or *Lactobacili* (a gift from Dr. Alain Stintzi) incubated for 37°C for 60 minutes. The antimicrobial capacity of crypt-culture extracts was assessed by counting the overnight growth of WT and *Sigirr* -/- crypt-treated *E. coli* or *Lactobacili*, and expressed as a percentage of the growth observed in untreated cultures.

2.2.9 FITC intestinal permeability assay

The assay was performed as previously described [208]. Uninfected or infected mice at D6 pi were gavaged with 150 μl of 80 mg/ml 4 kDa FITC-dextran (Sigma; FD4) in PBS 4 hrs prior to sacrifice. Mice were anaesthetized and blood was collected by cardiac puncture, which was added immediately to a final concentration of 3% acid-citrate dextrose (20 mM citric acid, 100 nM sodium citrate, 5 mM dextrose). Plasma was collected and fluorescence was quantified using a VictorX3 (Perkin-Elmer Life Sciences) at excitation 485 nm, emission 530 nm for 1 sec.

2.2.10 Measurement of IL-1β in tissues

Tissues were collected as described above. After homogenization and centrifugation, tissue supernatants were collected and IL-1β levels were assessed using an ELISA kit (BD Bioscience) following manufacturer’s instructions.

2.2.11 Western blotting

Following isolation of intestinal crypts, cells were lysed in RIPA buffer on ice. IEC proteins (50μg) were resolved by 12% SDS-PAGE and transferred to PVDF membranes. Blots were
blocked for 1 h with 5% milk in TBST. Membranes were incubated with primary antibody in TBST overnight at 4°C and probed with the respective secondary antibody for 1 hr at room temperature. Rabbit polyclonal IL-1β (Genetex) and mouse polyclonal actin (SantaCruz) antibodies were used in this study.

2.2.12 Statistical analysis

All results are expressed as the mean value ± SEM. Unless specified otherwise, non-parametric Mann–Whiney t-tests, or Student t tests were performed (Prism version 4.00). A P value of 0.05 or less was considered significant.

2.2.13 Gene accession numbers

The following are the GeneIDs (Database: Entrez Gene) for each gene analyzed in this manuscript, given as gene name (official symbol GeneID #): TNF-α (Tnf GeneID: 21926); IL-1β (Il1b GeneID: 16176); IFN-γ (Ifng GeneID: 15978); IL-17A (Il17a GeneID: 16171); MCP-1 (Ccl2 GeneID: 20296); iNOS (Nos2 GeneID: 18126); mCRAMP (Camp GeneID: 12796); MIP2 (Cxcl2 GeneID: 12796); Reg-3γ (Reg3g GeneID: 19695); SIGIRR (SIGIRR GeneID: 24058)
2.3 Results:
2.3.1 MyD88 signaling in IEC plays little role in the host response to *C. rodentium* infection
MyD88 signaling plays a critical role in the development of protective host responses to *C. rodentium* infection, controlling pathogen burdens, driving inflammation and promoting IEC integrity/proliferation [200,201]. As a result, *Myd88* -/- mice are highly susceptible to *C. rodentium* infection, carrying heavy pathogen burdens and suffering severe mucosal damage and loss of IEC barrier function (Figure 2.1A to 2.1D). Since many of these MyD88 dependent protective responses involve changes in IEC function, I tested whether they reflected MyD88 signaling within the IEC. Crossing MyD88 flox mice with mice expressing the cre enzyme under the villin (IEC specific) promoter, I generated IEC-*Myd88* -/- mice that were then infected with *C. rodentium* along with control *Myd88* flox mice. Interestingly, MyD88 signaling within IEC had little impact on the host response to infection. Pathogen burdens in the IEC-*Myd88* -/- mice were similar to those in control mice (Figure 2.1A), as were crypt heights and tissue integrity. Moreover, I noted no overt difference in barrier function or inflammatory cell recruitment to the infected cecum between these two mouse strains (Figure 2.1B to 2.1D). These data indicate that MyD88 signaling within IEC plays little role in driving the innate host response to *C. rodentium* infection.
Figure 2.1: MyD88 signaling in IEC is not required for protection against C. rodentium infection. Myd88 -/-, Myd88 flox and IEC-Myd88 -/- mice were infected for 6 days with C. rodentium. Infected IEC-Myd88 -/- mice carried similar (A) pathogen burdens, (B) levels of serum FITC, and (C-D) similar mucosal damage in the ceca as Myd88 flox mice. Moreover all of these readouts are significantly greater in Myd88 -/- mice, as compared to Myd88 flox and IEC-Myd88 -/- mice. Pathogen counts represent mucosal associated bacteria. Results are pooled from 2 independent infections with n=3-4 mice per group. Error bars = SEM, (Student t test *P< 0.05, ** P<0.01). Images were taken at 200x magnification.
2.3.2  *Sigirr* -/- mice develop exaggerated colitis during *C. rodentium* infection

While it is unclear why IEC play such a limited role in driving MyD88 dependent responses to *C. rodentium* infection and other forms of colitis [179,215,216], we recently showed that SIGIRR, a negative regulator of TLR and IL-1R signaling, is expressed by IEC and limits their responses to bacterial PAMPs [192]. To test the impact of SIGIRR *in vivo, Sigirr* -/- mice were infected with *C. rodentium* under co-housing conditions (WT and *Sigirr* -/- mice in same cage) or with WT and *Sigirr* -/- mice housed in different cages, and similar results were obtained. As shown in Figure 2.2A, infected WT mice did not display weight loss at day (D) 2 post-infection (pi) followed by progressive weight gain. In contrast, infected *Sigirr* -/- mice exhibited significantly greater (P<0.05) weight loss (~10%) starting from D4 until D10 pi (Figure 2.2A). Following their euthanization, the *Sigirr* -/- mice showed greater macroscopic signs of infection than WT mice, with overt loss of stool content and edema seen throughout their large intestines (Figure 2.2B). Moreover, pus and blood were often found within the ceca of infected *Sigirr* -/- mice and between 60% and 80% of these mice suffered macroscopic ulcers at D6 and D10 pi, a phenotype not observed in WT mice.
Figure 2.2: Sigirr −/− mice suffer more severe cecitis during C. rodentium infection.

Sigirr −/− mice exhibited (A) rapid weight loss by D4 pi. At both D6 and D10 pi, (B-C) their ceca displayed severe damage, with loss of stool contents and focal ulcers. (D) Cecal tissues from Sigirr −/− mice had significantly higher pathology scores at D6 and D10 pi compared to WT mice. (E) Plating revealed Sigirr −/− mice carried significantly higher pathogen burdens than WT mice in (E) cecal and colonic tissues, but their burdens were similar in (F) liver or spleens. Pathogen counts represent mucosal associated bacteria. Results are pooled from 2 independent infections with n=3-4 per group. Error bars = SEM, (Two-way ANOVA (Figure A), Student t test (Figure D, E, and F, *P < 0.05, **P< 0.01). Images were taken at 200x magnification.
Based on the observed pathology, as well as the fact that baseline SIGIRR gene transcript levels in WT mice were higher in the cecum than in the colon (Figure 2.3), I focused my subsequent analysis on the cecum. At both D6 and D10 pi, Sigirr -/- mice showed significantly higher cecal pathology scores compared to WT mice, reflecting increased crypt hyperplasia, edema, and greater inflammatory cell infiltration (Figure 2.2C to 2.2D, P<0.05). Specifically, I noted increased macrophage and neutrophil infiltration into the cecal mucosa of Sigirr -/- mice.

![SIGIRR expression in tissue](image)

**Figure 2.3: SIGIRR expression in cecal and colonic tissue of C57BL/6 mice.**
Cecal and colonic tissues were collected from WT mice at baseline and Sigirr expression level was assessed by qPCR. Sigirr expression level in the cecum is higher compared to expression in the colon. (n=5, * P<0.05, Paired t test)

Similarly, gene transcript levels for chemokines (MCP-1 and MIP-2α), antimicrobial factors (mCRAMP, β-defensin III, iNOS, and Reg 3-γ) and cytokines (IL-17A, TNF-α and IFN-γ)
were all significantly elevated at D6 and D10 pi in Sigirr -/- mice compared to WT mice. (Figure 2.4)

![Gene expression D6 and D10 pi](image)

Figure 2.4: Sigirr -/- mice exhibit higher inflammatory responses than WT mice during C. rodentium infection.

Quantitative PCR of cecal tissues from WT and Sigirr -/- mice at D6 and D10 pi revealed Sigirr -/- mice undergo significantly greater increases in gene transcript levels for pro-inflammatory chemokine, cytokine and anti-microbial genes. Results are pooled from 2-3 individual infections with n=3-5 per group. Error bars = SEM, (Student t test, *P< 0.05)

Based on their exaggerated colitic responses, I expected Sigirr -/- mice would show some level of protection against infection. Instead, they carried significantly higher (10-100 fold) C. rodentium burdens in their ceca and colons compared to WT mice at both D6 and D10 pi (Figure 2.2E, P<0.01). Interestingly, these higher burdens reflected increased numbers of C. rodentium infecting the intestinal mucosal surface, as well as within the intestinal lumen.
Notably, despite their high intestinal pathogen burdens, Sigirr-/- mice did not show increased susceptibility at systemic sites, as C. rodentium burdens in the spleens and livers of Sigirr-/- mice were similar to those in WT mice (Figure 2.2F).

2.3.3 Sigirr-/- mice exhibit a hyper-proliferative IEC response against C. rodentium infection

Despite carrying heavy C. rodentium burdens and suffering exaggerated colitic damage, Sigirr-/- mice survived infection and healed their mucosal ulcers by D14 pi, suggesting intestinal mucosal repair was enhanced in these mice. I assessed IEC proliferation in Sigirr-/- and WT mice (by Ki-67 staining) to examine their ability to repair mucosal damage and also measured IEC barrier integrity. Increased IEC proliferation was evident by D4 pi in the Sigirr-/- mice, but not in WT mice. By D6 pi, IEC proliferation in Sigirr-/- mice was significantly greater than in WT mice (Figure 2.5A to B, 75 ± 7% vs 41 ± 4%, P<0.01) and similar results were observed at D10 pi. While I also assessed IEC barrier function by oral FITC gavage, I noted no differences in serum levels of FD4 between infected Sigirr-/- mice and WT mice (Figure 2.5C).
Figure 2.5: Sigirr -/- mice exhibit stronger inflammatory responses during C. rodentium infection.

Immunostaining for the proliferation marker (A) Ki-67 (red) revealed Sigirr -/- mice exhibit increased IEC proliferation in cecal tissues by D4 pi. (B) At D6 and D10 pi, there are significantly more proliferating IEC in Sigirr -/- mice compared to WT mice. (C) WT and Sigirr -/- mice suffer similar levels of barrier permeability following infection. (D) Sigirr -/- mice carry significantly higher gene transcript levels for antimicrobial peptides and chemokines compared to WT mice following cecal loop surgery. Results are representative of 4 independent infections with n=3-4 per group. Error bars = SEM, (Student t test (Figure B and C), Mann-Whitney t test (Figure D), (*P< 0.05, **P< 0.01)
2.3.4 The negative regulator SIGIRR suppresses the host response to *C. rodentium* infection

I next examined whether SIGIRR impacted very early host responses to *C. rodentium* by employing the cecal ligation model of acute *C. rodentium* infection, allowing me to inject equal pathogen numbers into the ceca of WT and Sigirr -/- mice. As expected, similar numbers of *C. rodentium* were found infecting the cecal tissues of WT and Sigirr -/- mice after 10 hr of infection (WT: 1.9± 0.7 x 10^8 CFU/g, Sigirr -/-: 1.2± 0.5 x 10^8 CFU/g). Cecal tissues were collected and qPCR was used to determine transcript levels for genes encoding several antimicrobial peptides and other IEC derived factors. As shown in Figure 2.5D, infection of WT mice led to a modest increase in the transcription of genes encoding antimicrobial peptides (*mCRAMP, Reg3-γ, β-defensin III*) and chemokines (*MCP-1 and MIP-2α*). Strikingly, I observed elevated transcription of most genes in Sigirr -/- infected mice (P<0.05), despite the two mouse strains carrying similar pathogen burdens. These results indicate that in the absence of SIGIRR, the host intestine (likely IEC) responds very rapidly to *C. rodentium* infection, and that the exaggerated colitis suffered by infected Sigirr -/- mice is largely dependent on their lack of SIGIRR, rather than differences in pathogen burdens.

2.3.5 SIGIRR’s actions reflect its expression by non-BM derived cells

SIGIRR thus plays a critical role in suppressing *C. rodentium* induced colitis, but the cellular source of SIGIRR is unclear. SIGIRR is expressed by IEC, as well as by several BM-derived cell types including dendritic cells and T cells. To better define the cellular source of SIGIRR that is responsible for observed phenotype during *C. rodentium* infection, Dr. Kelly McNagny and Dr. Michael Hughes generated hematopoietic chimeras by BM transplantation between Sigirr -/- mice and WT mice expressing Ly5.1 on their BM-derived cells. Following
infection by *C. rodentium*, *Sigirr* -/- + WT BM chimeric mice developed severe colitis, similar to that seen in *Sigirr* -/- mice. Moreover the ceca of infected WT + *Sigirr* -/- BM mice were macroscopically similar to those of WT mice, although histologically, they displayed a greater increase in crypt lengths (Figure 2.6B - D) than WT mice. Pathology scoring also revealed *Sigirr* -/- + WT BM mice had exaggerated crypt hyperplasia, edema and immune cell infiltration when compared to WT + *Sigirr* -/- BM mice (Figure 2.6D, P<0.05). Similar to *Sigirr* -/- mice, *Sigirr* -/- + WT BM chimeras carried significantly higher intestinal *C. rodentium* burdens, compared to WT mice and WT + *Sigirr* -/- BM mice at D10 pi (Figure 2.6A). Furthermore, *Sigirr* -/- + WT BM mice exhibited hyper-proliferative IEC responses (65 ± 5%) similar to those of *Sigirr* -/- mice (72 ± 6%), as revealed by Ki-67 staining while the IEC proliferative response was similar between WT mice (39 ± 4%) and WT + *Sigirr* -/- BM mice (33 ± 3%) (Figure 2.6E to F). Thus expression of SIGIRR by non-BM derived cells (putative IEC) plays the major role in suppressing the host inflammatory response and controlling *C. rodentium* burdens. Moreover, loss of SIGIRR in this compartment leads to the exaggerated colitis suffered by *C. rodentium* infected *Sigirr* -/- mice.
Figure 2.6: Non-BM derived cells mediate SIGIRR-dependent mucosal responses. WT (Ly 5.1) and Sigirr-/- (Ly 5.2) mice (Both of C57BL/6 background) were used to generate BM chimeric mice, which were then infected for 10 days with C. rodentium. Only mice exhibiting greater than 90% chimerism were infected. Similar to Sigirr -/- mice, Sigirr -/- + WT BM mice displayed significantly heavier (A) pathogen burdens compared to WT mice. The ceca of Sigirr -/- + WT BM mice displayed (B) severe macroscopic and (C) histologic damage with significantly (D) greater pathology scores compared to WT and WT + Sigirr -/- BM mice. Sigirr -/- + WT BM mice exhibit higher levels of IEC proliferation as revealed by (E and F) Ki-67 staining. Pathogen counts represent mucosal associated bacteria. Results are pooled from 2 independent infections with n=3-4 per group. Error bars = SEM, (Student t test (Figure A and D), One way ANOVA with Bonferroni post test for (Figure F), *P< 0.05, **P< 0.01). Images were taken at 200x magnification.
2.3.6 Survival of infected *Sigirr* -/- mice requires MyD88, but not TLR2 or TLR4 signaling

SIGIRR is a negative regulator of TLR and IL-1R signaling, but the identity of the (dysregulated) receptor(s) that drive the exaggerated colitis in infected *Sigirr* -/- mice is unclear. To identify the receptor(s) involved, we began by removing MyD88 dependent signaling from *Sigirr* -/- mice. We crossed *Myd88* -/- mice and *Sigirr* -/- mice and infected the resulting *Myd88/Sigirr* -/- mice. Similar to *Myd88* -/- mice, *Myd88/Sigirr* -/- mice became sick and required euthanization by D6-8 pi (Figure 2.7A). They also carried heavy *C. rodentium* burdens (~10^{10} CFU/g) at D6 pi and suffered severe mucosal damage and increased intestinal permeability much like *Myd88* -/- mice (Figure 2.7), confirming SIGIRR’s actions in this model depends on MyD88 signaling.
Figure 2.7: Myd88/Sigirr -/- suffers from extensive mucosal injury and increased barrier permeability

Myd88 -/-, Myd88/Sigirr -/- and Sigirr -/- mice were infected for 6 days with C. rodentium. Myd88/Sigirr -/- mice exhibited significant increased barrier permeability and sustained severe mucosal damage in the cecum compared to Sigirr -/- mice. Results are pooled from 2 independent infections with n=3-4 per group. Error bars = SEM, One way ANOVA with Bonferroni post test for (Figure A), *P< 0.05). Images were taken at 200x magnification.

Next, I sought to identify the specific TLRs responsible for the exaggerated colitis seen in Sigirr -/- mice. Previous studies have shown that the host’s response to C. rodentium infection is largely dependent on TLR2 and TLR4 [203,204]. Therefore, I crossed Tlr2 -/- mice and Tlr4 -/- mice with Sigirr -/- mice to generate Tlr2/Sigirr -/- mice and Tlr4/Sigirr -/- mice. Upon infecting these mice along with WT and Sigirr -/- mice, all mouse strains were found to survive the infection (Figure 2.8A). As expected, all readouts were dramatically greater in the Sigirr -/- mice, as compared to WT mice. Interestingly, the responses in Tlr2/Sigirr -/- mice and Tlr4/Sigirr -/- mice were similar to those in the Sigirr -/- mice.
including pathogen burdens, and pathological damage (Figure 2.8B to C). Hence, the data suggest that the exaggerated damage and increased pathogen burden observed in the Sigirr -/- mice are largely independent of TLR2 and TLR4.
Figure 2.8: MyD88 signaling is required for the survival of infected Sigirr -/- mice. WT, Sigirr -/-, Myd88/Sigirr -/-, Tlr2/Sigirr -/-, and Tlr4/Sigirr -/- were infected by C. rodentium for 6 and 10 days. (A) Myd88/Sigirr -/- mice required euthanization by D8 pi whereas the other mouse groups survived the infection. (B) All mice on a Sigirr -/- background carried significantly heavier pathogen burdens and (C) showed more severe colitis compared to WT mice at D6 and D10 pi. (C-D) Immunostaining for the proliferation marker Ki-67 demonstrated infected Sigirr-/-, Tlr2/Sigirr -/- and Tlr4/Sigirr -/- display elevated IEC proliferation compared to WT mice. (E) Phospho STAT-3 staining is restored in Tlr2/Sigirr -/- mice, while (F) the heightened barrier disruption seen in infected Tlr2-/- mice is normalized in Tlr2/Sigirr -/- mice. Pathogen counts represent mucosal associated bacteria. Results are pooled from 2-3 independent infections, each with n=3-4 per group. Error bars = SEM, (Student t test (Figure B, D) and one way ANOVA (Figure D), *P< 0.05, **P< 0.01). Images were taken at 200x magnification.
2.3.7 SIGIRR controls IEC proliferation and integrity largely independent of TLR2 and TLR4

In previous studies, our laboratory showed TLR2 and TLR4 play specific and critical roles in controlling IEC responses during *C. rodentium* infection [208]. TLR4 signaling drives IEC proliferation during infection, while TLR2 signaling promotes IEC integrity. In fact, infected *Tlr2* -/- mice suffer exaggerated IEC barrier permeability, and impaired phospho STAT3 signaling within their IEC, with these defects leading to cecal and colonic ulcers and high mortality rates [203]. When I assessed IEC responses in the *Tlr2/ Sigirr* -/- mice and *Tlr4/Sigirr* -/- mice, the *Tlr2/Sigirr* -/- mice exhibited similar proliferative responses (74 ± 7%, Figure 5D) to *Sigirr* -/- mice (75 ± 7%), as demonstrated by Ki-67 staining. Interestingly, *Tlr4/Sigirr* -/- mice showed an intermediate level of IEC proliferation (58 ± 3%), modestly reduced compared to *Sigirr* -/- mice, yet still significantly greater (P<0.05) than WT mice (36 ± 4%). This data suggests that signaling through other SIGIRR-regulated receptors drives IEC proliferation in *Sigirr* -/- mice, even in the absence of TLR4.

I next tested barrier function in these mice (and *Tlr2* -/- mice). At D6 pi, FITC serum levels within *Tlr2* -/- mice were significantly elevated compared to WT mice, confirming their impaired barrier function (Figure 2.8F, P<0.05). Similar to WT mice, *Sigirr* -/- mice showed no overt barrier dysfunction, but strikingly, the concurrent loss of SIGIRR (*Tlr2/Sigirr* -/- mice) compensated for the barrier dysfunction seen in *Tlr2* -/- mice. Furthermore, the impaired phospho STAT-3 staining seen in *Tlr2* -/- mice was restored in *Tlr2/Sigirr* -/- mice (Figure 2.9E). These results indicate that loss of SIGIRR leads to IEC responses that promote mucosal integrity in a manner largely able to compensate for the defects caused by TLR2 or TLR4 deficiency, suggesting another SIGIRR regulated receptor is at play.
2.3.8 Exaggerated IEC responses in Sigirr-/- mice require IL-1R signaling

Aside from negatively regulating TLRs, SIGIRR also negatively regulates IL-1R signaling [186,187,198]. I assessed the expression of its ligands IL-1α and IL-1β. Interestingly, IL-1α gene expression showed a dramatic increase in the cecal tissues of Sigirr-/- mice during infection (Figure 2.9A, P<0.05). Moreover protein analysis by ELISA revealed significantly increased IL-1β levels in Sigirr-/- mice compared to WT mice under both baseline and infected conditions (Figure 2.9 B and C, P<0.05). Similarly, cecal crypts isolated from Sigirr-/- mice showed elevated IL-1β levels under baseline and infected conditions as measured by Western blot (Figure 2.9C). To address the potential role of IL-1R signaling in the response of the Sigirr-/- mice, we generated Il-1r/Sigirr-/- mice and infected them with C. rodentium. The Il-1r/Sigirr-/- mice rapidly lost weight during infection, requiring their euthanization by D10 pi (Figure 2.9D).
Figure 2.9: IL-1R signaling is required for the exaggerated IEC responses in Sigirr −/− mice.

The cecal tissues from infected Sigirr −/− mice show increased abundance of IL-1α gene transcripts (A) as compared to WT mice. The Sigirr −/− mice also express increased levels of IL-1β protein in their cecal tissues under uninfected and C. rodentium infected conditions as measured by (B) ELISA and (C) Western blot. Il-1r/Sigirr −/− mice suffer increased (D) mortality rates, (E) elevated pathogen burdens and (F) severe mucosal damage. The severe damage suffered by the Il-1r/Sigirr −/− mice was accompanied by impaired IEC proliferation as shown by (G) immunostaining for Ki-67 and by higher IEC permeability as quantified by (H) FD4 in serum. Pathogen counts represent mucosal associated bacteria. Results are pooled from 2-4 independent infections with n=3-4 per group. Error bars = SEM, (Student t test (Figure A, B, E), one-way ANOVA (Figure G, H), *P< 0.05, **P< 0.01). Images were taken at 200x magnification.
At D6 pi, we noted that although the *Sigirr* -/- mice, *Il-1r* -/- mice and *Il-1r/Sigirr* -/- mice all carried similar intestinal pathogen burdens, the *Il-1r/Sigirr* -/- mice suffered extensive mucosal damage compared to the *Sigirr* -/- mice and the *Il-1r* -/- mice (Figure 2.9E to F). Remarkably, the protective, hyper-proliferative IEC responses observed in the *Sigirr* -/- mice were absent in the *Il-1r/Sigirr* -/- mice (Figure 2.9F), with almost 3 fold more IEC staining positively for Ki-67 in the *Sigirr* -/- mice compared to the *Il-1r/Sigirr* -/- mice (72 ± 6% vs 26 ± 5%, P<0.01). Moreover, infected *Il-1r/Sigirr* -/- mice suffered significantly greater intestinal permeability compared to *Sigirr* -/-mice as measured by serum FD4 levels (Figure 2.9G, P<0.01), whereas the *Il-1r* -/- mice showed serum FD4 levels that were intermediate between the two other mouse strains. These findings indicate that the exaggerated and protective IEC responses seen in *Sigirr* -/- mice are largely IL-1R dependent and IL-1R may be the key receptor regulated by SIGIRR in this model.

### 2.3.9 SIGIRR deficiency increases susceptibility to *C. rodentium* colonization/infection

While *Sigirr* -/- mice develop exaggerated colitic and IEC proliferative/reparative responses during infection, these mice also appear more susceptible to *C. rodentium* colonization, carrying significantly higher pathogen burdens than mice expressing SIGIRR. To test whether *Sigirr* -/- mice are truly more susceptible to *C. rodentium* infection, I infected WT and *Sigirr* -/- mice with a 100 fold lower dose (LD) of *C. rodentium* and followed their course of infection by plating feces. Previous studies have found this low dose is unable to effectively infect WT mice [217], and indeed, LD WT mice shed only low numbers of *C. rodentium* (10^4-10^6 CFU/g of feces). In contrast, LD *Sigirr* -/- mice showed significantly (100x) higher *C. rodentium* burdens by D2 pi and their pathogen burdens continued to
increase until D6 pi at ~ $2.6 \times 10^9$ CFU/g of feces, similar to the burdens carried by $\textit{Sigirr}^{-/-}$ mice given a full infectious dose (Figure 2.10A). At D10 pi, following tissue collection, $\textit{Sigirr}^{-/-}$ mice were found to carry 1000 fold more $C. \textit{rodentium}$ in both cecal and colonic tissues compared to WT mice (Figure 2.10B, P<0.01). As expected from their pathogen burdens, $\textit{Sigirr}^{-/-}$ mice developed severe colitis and cecal pathology similar to that seen with a full dose infection while WT mice did not display any overt or histological signs of infection or pathology (Figure 2.10B).
Figure 2.10: *Sigirr* -/- mice are highly susceptible to enteric infection.

(A) *Sigirr* -/- mice were heavily colonized by 100x lower dose (LD) of *C. rodentium* by D2 pi. By D10 pi, *Sigirr* -/- mice carried 1000x heavier pathogen burdens and developed severe mucosal damage compared to WT mice. (B) WT and *Sigirr* -/- mice infected by *S. Typhimurium* without streptomycin pre-treatment. By D7 pi, *Sigirr* -/- mice were heavily colonized and suffered extensive cecal injury. Pathogen counts represent mucosal associated bacteria. Results are pooled from 2-3 independent infections with n=3 per group. Error bars = SEM, (Two-way ANOVA (Figure A), Student t test (Figures B and C), *P< 0.05, **P< 0.01)). Histological images were taken at 100x magnification.
2.3.10 *Sigirr* -/- mice are also highly susceptible to *S. Typhimurium* infection
Thus *Sigirr* -/- mice show unusual susceptibility to *C. rodentium* colonization and infection. I next tested whether they showed heightened susceptibility to infection by another enteric bacterial pathogen. I orally inoculated WT and *Sigirr* -/- mice with the enteric bacterial pathogen *S. Typhimurium*. *S. Typhimurium* is known to poorly colonize the intestines of mice, in large part because it is unable to displace competing commensal microbes [126,127]. As a result, pretreating mice with the antibiotic streptomycin is commonly used to remove competing commensals and facilitate *S. Typhimurium* colonization and infection of the cecum. While I found *Sigirr* -/- mice were more susceptible than WT mice to *S. Typhimurium* infection following streptomycin pretreatment at early time points (Figure 2.11), I also tested their susceptibility to *S. Typhimurium* in the absence of antibiotic pretreatment. As expected, oral inoculation of WT mice with a dose of 5 x 10^6 CFU of ΔaroA *S. Typhimurium* did not lead to any significant cecal pathology or colonization by this pathogen. In contrast, the same dose led to dramatically heavier (1000 fold) *S. Typhimurium* burdens in the cecum and severe inflammation and pathology in the ceca of *Sigirr* -/- mice (Figure 2.10C). ΔaroA *S. Typhimurium* were used since C57BL/6 mice are highly susceptible to *S. Typhimurium* infection because they are Nramp negative [218] Taken together, these results show that loss of SIGIRR expression leads to dramatically enhanced susceptibility to oral infection by enteric bacterial pathogens, in a manner able to overcome the typical commensal microbe dependent resistance to such colonization.
Figure 2.11: *Sigirr* -/- mice suffer more severe colitis during ΔaroA *Salmonella Typhimurium* infection with streptomycin pre-treatment.

*Sigirr* -/- mice carried significantly higher pathogen burdens at D3 pi (left) and suffered from severe mucosal damages in the cecum (* P<0.05). n=4, Mann Whitney T test.

2.3.11 *Sigirr* -/- mice undergo rapid commensal microbe depletion following infection

Previous studies by our group and others found that differences in the makeup of intestinal microbiota can affect host susceptibility to *C. rodentium* infection [219]. Previous sequencing studies found no overt differences between the microbiota of *Sigirr* -/- mice and WT mice [220], but considering the susceptibility of *Sigirr* -/- mice to enteric pathogens, I speculated they might instead carry fewer total commensal microbes than WT mice. I enumerated the microbiota in the feces of these mice by fluorescence microscopy under baseline conditions, but noted no significant differences in commensal numbers between WT mice and *Sigirr* -/- mice (Figure 2.12A). Through qPCR and fluorescence in situ hybridization (FISH) analysis, I confirmed previous reports by Chan et al. (2011), that *Sigirr* -/- mice showed no major differences in the makeup of their various commensal microbial species and phyla compared to WT mice (Figure 2.12B to C).
Figure 2.12: Sigirr -/- mice display strong antimicrobial activity against commensal microbes.

No significant differences were found between (A) the commensal microbiota found in WT and Sigirr -/- mice as measured by (B) qPCR and (C) FISH staining. Rapid commensal depletion in Sigirr -/- mice was observed as early as D1 pi (D) while intestinal crypts isolated from Sigirr -/- mice (E) possess greater killing activity against commensal E. coli and Lactobacilli. Results are pooled from 2-3 independent experiments (or infections) with n=3-5 per group. Error bars = SEM, (Student t test (Figure A) one way ANOVA (Figures B-F, *P< 0.05, **P< 0.01)
Interestingly, Lupp et al. [64], showed that *C. rodentium* infection causes a dramatic shift in the makeup of the gut microbiota as well as a reduction in total commensal numbers. These commensal changes were host mediated and took approximately 6 days to occur, corresponding with the time taken for *C. rodentium* to heavily colonize its host. Our group, and others have speculated these changes in the microbiota may facilitate *C. rodentium* colonization, as antibiotic based removal of commensals accelerates the course of *C. rodentium* infection [219,221]. I quantified commensal populations within the ceca of infected WT mice, and confirmed it takes approximately 6 days for the aforementioned commensal depletion (80%) to occur. In contrast, commensal numbers in the Sigirr -/- mice dropped dramatically by D1 pi, and this reduction remained evident until D6 pi and beyond (Figure 2.12D). The corresponding *C. rodentium* burdens increased in concert (albeit slightly delayed) with the loss of commensals. Similar rapid changes in commensals were also detected in *Sigirr -/-* mice following infection with LD *C. rodentium* (Figure 2.13). Interestingly, I did not observe this commensal depletion when *Sigirr -/-* mice were infected with an avirulent strain (ΔescN) of *C. rodentium* (data not shown). These results suggest the impaired resistance to pathogen colonization I observed in *Sigirr -/-* mice reflects the ability of even very low doses of *C. rodentium* (and likely other pathogens) to rapidly trigger commensal depletion in these mice, thereby opening niches within the intestine for rapid pathogen colonization.
Figure 2.13: Rapid removal of commensal bacteria in Sigirr -/- is dependent on virulence factor and not pathogen number.
Sigirr -/- mice exhibit rapid commensal depletion by D1 pi after LD C. rodentium infection.

2.3.12 Sigirr -/- crypt epithelial cells show heightened antimicrobial activity
I next addressed the basis for the rapid depletion of commensal microbes seen in infected Sigirr -/- mice. Notably, the massive commensal depletion occurs prior to overt intestinal inflammation. As outlined in Figure 2.8D, acute exposure to C. rodentium rapidly increased anti-microbial gene expression, likely reflecting responses by IEC. I thus examined whether the IEC of Sigirr -/- mice harbored greater bactericidal activity than WT mice towards commensal bacteria, using E. coli and Lactobacilli species as examples. I isolated intestinal crypts from uninfected WT and Sigirr -/- mice; collected their crypt supernatants and tested their bactericidal activity against commensal microbes. As shown in Figure 2.12E, after 1hr incubation, isolates from Sigirr -/- mice showed a killing activity of 29.9 ± 3.5 % against E. coli while crypt isolates from WT mice showed only 10.6 ± 9.9%. Similarly, isolates from Sigirr -/- mice displayed greater killing activity against Lactobacilli, a Gram-positive microbe (Figure 2.12F, P<0.05). Therefore, crypt isolates from Sigirr -/- mice possess significantly higher baseline bactericidal activity than those from WT mice. Considering the
rapid induction of antimicrobial genes seen in the cecal loop model, the heightened antimicrobial activity of Sigirr -/- mice likely explains the rapid commensal depletion seen following their infection.
2.4 Discussion

MyD88 dependent signaling plays a critical protective role during *C. rodentium* infection, promoting inflammatory and antimicrobial responses that control *C. rodentium* burdens, as well as homeostatic responses that protect IEC barrier function and limit/repair IEC injury [200,201]. While many of these responses involve changes in IEC function, I clearly show that MyD88 signaling within IEC plays little role in driving these responses. Rather than IEC being unable to respond to *C. rodentium* infection, I instead show that the negative regulator SIGIRR suppresses their responsiveness. While *Sigirr* -/- mice react strongly to pathogenic insults to the GI tract, *Sigirr* -/- mice do not develop spontaneous colitis or other baseline pathologies, but instead show a modestly increased inflammatory tone within their intestines [195]. When I infected *Sigirr* -/- mice, they developed exaggerated inflammatory and antimicrobial responses as well as increased IEC proliferation and repair. These responses began within the IEC layer, and through BM reconstitution studies, I confirmed that it was SIGIRR expression by non-BM cells (putative IEC) that limits the host inflammatory and IEC responses to *C. rodentium*. This data thus expand our previous *in vitro* studies that the innate responsiveness of IEC to A/E bacterial pathogens is dramatically elevated in the absence of SIGIRR.

In collaboration with Dr. Li’s group, we previously showed *Sigirr* -/- mice develop exaggerated DSS colitis and IEC proliferation that was commensal microbe dependent, but it was unclear which SIGIRR regulated receptors drove these responses. In the current study, I tested the role of MyD88, as well as individual receptors and as expected, loss of MyD88 left *Sigirr* -/- mice suffering severe mucosal damage and rapidly succumbing to infection, much like *Myd88* -/- mice that express SIGIRR. As previously mentioned, TLR4 signaling drives
the majority of inflammation and IEC proliferation during *C. rodentium* infection of SIGIRR expressing mice [208]. Similarly TLR2 signaling in these mice is required to protect and repair IEC barrier function during infection. Interestingly, neither TLR2 nor TLR4 were required for these roles in the absence of SIGIRR. In fact, SIGIRR deficiency led to increased IEC proliferation/barrier protection that was largely able to compensate for the defects caused by either TLR2 or TLR4 deficiency, suggesting another receptor was responsible for the protective IEC responses seen in Sigirr -/- mice.

My data indicate that IL-1R signaling drives the exaggerated IEC responses seen in Sigirr -/- mice. While IL-1R signaling plays a modest protective role during *C. rodentium* infection of SIGIRR expressing hosts [147], it appears to be primarily responsible for the heightened IEC proliferation and barrier protection seen in Sigirr -/- mice, as these exaggerated IEC responses were lost in Il-1r/Sigirr -/- mice. In the context of the heavy pathogen burden and heightened inflammation seen in the Sigirr -/- mice, IL-1R signaling proved crucial in limiting the severity of the resulting mucosal damage and ultimately for the survival of infected Sigirr -/- mice. Interestingly, I found elevated levels of both IL-1α and IL-1β in the intestinal tissues of Sigirr -/- mice under both baseline and infected conditions relative to wildtype mice. Both IL-1α and IL-1β have been shown to induce IEC proliferation *in vitro*, as well as induce expression of chemokines and antimicrobial factors by IEC [222,223,224]. At present it is unclear which cytokine (or perhaps both) drives the IL-1R dependent responses seen in the Sigirr -/- mice.

My data also indicate it is primarily SIGIRR expression by non- BM derived cells that limits the host response to *C. rodentium* infection. While several BM derived cell types
(macrophages, dendritic cells, T cells) found in the intestine express SIGIRR, the only described non-BM derived cell type is the IEC [186,199]. It has been speculated that maintaining the innate hypo-responsiveness of IEC helps prevent spontaneous commensal microbe driven intestinal inflammation, as seen in patients with IBD. While this may be the case in some situations, my data suggest that SIGIRR instead controls the threshold at which IEC respond to invading bacterial pathogens.

Notably, despite their innate hyper-responsiveness, Sigirr -/- mice proved highly susceptible to infection, undergoing very rapid pathogen colonization and ultimately carrying significantly heavier C. rodentium burdens than WT mice. Unlike Myd88 -/- mice where their immunodeficiency not only led to increased intestinal pathogen burdens, but also lethally high C. rodentium burdens in the liver and spleen, the Sigirr -/- mice only showed increased pathogen burdens in their ceca and colons. Heavy C. rodentium colonization of Sigirr -/- mice was evident between D2 and 4 pi, compared to the 6 days typically required for WT mice. Interestingly, we previously found that such rapid C. rodentium colonization was only seen in mice pretreated with the antibiotic streptomycin to displace commensal microbes [48]. Taken together with the susceptibility of Sigirr -/- mice to S. Typhimurium infection without the typical requirement for streptomycin pretreatment, we focused on potential defects in commensals as underlying the susceptibility of Sigirr -/- mice to pathogen colonization of their intestines.

As outlined by Chan et al. [220], deep sequencing identified few differences between Sigirr -/- mice and WT mice in their baseline intestinal microbiota. I also found they carried similar
numbers of intestinal commensal microbes, and showed only modest differences in their baseline microbiota makeup, as compared to WT mice. I therefore compared how the intestinal microbiota of Sigirr -/- and WT mice responded to C. rodentium infection. As shown by Lupp et al. [64], infection of WT mice leads to a host mediated depletion of the intestinal microbiota by D6 pi, concomitant with C. rodentium expansion and spread through the intestines of WT mice. I confirmed this timing in WT mice, whereas overt commensal depletion was found to occur by D1 pi in Sigirr -/- mice, and persisted thereafter. Interestingly, this rapid commensal depletion did not occur when Sigirr -/- mice were gavaged with avirulent C. rodentium mutant, suggesting it is a specific response to infection. While infected Sigirr -/- mice do not display overt colitis at the time of commensal depletion, we showed that acute exposure to C. rodentium dramatically elevated mRNA transcript levels of a number of antimicrobial genes within the ceca of Sigirr -/- mice. This increased antimicrobial tone had a functional effect, as intestinal crypt IEC isolates from even uninfected Sigirr -/- mice displayed significantly greater killing activity against commensal E. coli and Lactobacilli bacteria than IEC from WT mice. Thus the rapid depletion of commensal microbes seen in Sigirr -/- mice likely reflects the heightened antimicrobial activity of their IEC and provides the basis for their reduced colonization resistance against enteric pathogens.

Colonization resistance was initially described by van der Waaij et al. [68,210], as the process whereby the intestinal microbiota protects itself as well as the host against incursion by new and often harmful microbes. While there is significant evidence for colonization resistance, such as the hyper-sensitivity of germfree mice to enteric infections, the exact
mechanisms by which colonization resistance exerts its protective role are unclear [67,68,210]. Moreover, while such commensal mediated resistance to enteric pathogens is undoubtedly beneficial to the host, it is unclear whether host factors play a role in promoting colonization resistance. My data suggest that it is in fact the innate hypo-responsiveness of IEC that is critical in promoting colonization resistance. While it may seem counter-intuitive that limiting host anti-microbial and inflammatory responses in the GI tract would be protective, as our LD infection data show, in most cases, when the host is exposed to small numbers of enteric bacterial pathogens, the resident microbiota are able to prevent the pathogens from infecting the host. It appears that SIGIRR helps maintain host-commensal mutualism (and colonization resistance) in the face of noxious threats, such as the incursion by a bacterial pathogen, potentially by controlling the threshold at which the IEC respond.

My results are intriguing since the exaggerated inflammatory/anti-microbial responses in the intestines of Sigirr -/- mice appear to deplete only commensals and not C. rodentium. While this might suggest that the responses elicited in Sigirr -/- mice are poorly effective against C. rodentium (and S. Typhimurium), I believe the effects are more complex. It is well known that enteric bacterial pathogens utilize a number of strategies to subvert host defenses, including suppressing the inflammatory signaling of infected IEC [40], as well as possessing greater inherent resistance to the effects of antimicrobial peptides [225]. Moreover, recent studies have shown that enteric bacterial pathogens can utilize nutrients and metabolites released within the inflamed intestine that are unusable to commensal species [207,226]. Therefore the success of bacterial pathogens at replacing the microbiota in Sigirr -/- mice
may in fact demonstrate their evolutionary success at withstanding host anti-microbial responses in comparison to commensal microbes.

In conclusion, my data demonstrate that although SIGIRR suppresses host inflammatory, antimicrobial and IEC reparative responses, it actually plays a critical role in enteric host defense by promoting commensal based colonization resistance against enteric bacterial pathogens. Thus SIGIRR’s role in limiting the innate responsiveness of IEC is evidence of a complex defense strategy within the GI tract. This strategy delays the development of protective host responses to infection, by relying on cell types other than IEC to drive protective host (MyD88) responses to infection. However SIGIRR’s key role in promoting and protecting host mutualism with the gut microbiota has obvious advantages as well, preventing most infections by supporting the ability of the microbiota to resist invading microbes. While promoting colonization resistance to enteric infections is of great benefit, ultimately I believe that modulating SIGIRR expression within the intestine could also offer therapeutic potential for GI diseases, such as IBD. Increasing SIGIRR expression could potentially reduce inflammation in IBD patients, whereas suppressing SIGIRR could potentially help promote mucosal repair.
3 Chapter: The attaching/effacing bacterial effector NleC suppresses epithelial inflammatory responses by inhibiting NF-κB and p38-MAP Kinase activation

3.1 Introduction
EPEC and EHEC are among the most widespread bacterial causes of infantile diarrhea, both in developing and developed countries [227]. Despite the health risks posed by these attaching/effacing pathogens, there are few effective therapies to prevent or treat their infection. To develop improved therapies, we need a clearer understanding of how these bacteria successfully infect their hosts, particularly in the face of the varied host defenses found within the mammalian GI tract. Like other bacteria, EPEC and EHEC are recognized by innate host receptors such as TLRs and NOD-like receptors that detect conserved microbial molecular patterns [12,14,228]. This initial recognition enables the host to quickly respond to infectious threats by producing inflammatory cytokines and anti-microbial peptides [229,230,231,232,233]. In fact, these host inflammatory and immune responses to infection lead to intestinal tissue damage, including inflammatory cell infiltration into the infected mucosa and damage to the gut epithelium. Much of this pathology has been linked to inflammatory responses initiated by the infected IEC, including the production of the chemokine CXCL8 (formerly known as IL-8), an important neutrophil chemoattractant. Not surprisingly, enteric pathogens have evolved diverse and elegant strategies to avoid and suppress intestinal defenses in order to colonize and survive within the host’s GI tract. A common strategy involves the injection of bacterial proteins into host cells through a T3SS [234,235,236,237] with these effector proteins subverting key aspects of host cell function [227,230].
The mouse pathogen *Citrobacter rodentium* bears many similarities to EPEC [112], being non-invasive, infecting its hosts by attaching to the apical surface of intestinal epithelial cells, effacing their microvilli and creating pedestal-like structures, as well as causing colitis and mild diarrhea [76,114]. It also shares a similar array of translocated effectors with EPEC and EHEC. Several reports have shown that EPEC suppresses innate immune responses from infected IEC through the actions of its effectors [77,175,206]. These studies have demonstrated that IEC infected with WT EPEC produce lower levels of IL-8 as well as other inflammatory mediators when compared to cells infected by EPEC strains lacking a functional T3SS. The reduction in IL-8 release occurred in association with impaired NF-κB and p38 MAP kinase signaling. In part the suppression of IL-8 release appears to be mediated by the effector NleE, which was recently shown to inhibit NF-κB activation during EPEC infection [107] whereas the effector NleH1 was also found to subvert NF-κB function by preventing translocation of ribosomal protein S3 [108,109]. Since these effectors have only been shown to affect NF-κB signaling, I suspected that additional EPEC effectors were involved in inhibiting pro-inflammatory responses by targeting p38 MAP Kinase signaling pathways. Recently several studies have identified NleC as another effector that suppresses inflammatory response through inhibition of NF-κB [238,239,240,241]. These studies showed that NleC also suppressed IL-8 release and NF-κB activation by cultured epithelial cells, specifically through the degradation of the NF-κB p65 subunit. NleC is a zinc protease that cleaves the NF-κB p65 subunit [238,239,240,241]. Curiously, despite the prominent immunosuppressive role attributed to NleC *in vitro*, no studies have yet examined the potential for NleC to modulate inflammation *in vivo*. In addition, despite considerable
evidence that ectopic NleC suppresses IL-8 release by degrading the NF-κB p65 subunit, at least in vitro [238,239,240,241], attempts to localize the NleC protein within host cells has yielded conflicting results. While several studies identified ectopically expressed NleC as predominantly localized to the nucleus, where it could potentially access the NF-κB p65 subunit [238,239,240]; another study found that EPEC derived NleC was predominantly localized to the site of bacterial attachment, at the host cell membrane [241]. This discrepancy in NleC’s localization raised the possibility that NleC may impact other host signaling pathways, along with NF-κB. In this study, by screening an array of EPEC and C. rodentium mutants, I confirmed NleC as a novel effector protein involved in restraining epithelial inflammatory responses during infection, by suppressing NF-κB. I also found that it impacts p38 MAP kinase signaling and we demonstrate that NleC plays a significant role in suppressing the colitis triggered by C. rodentium infection, as well as impacting on the competitiveness of this pathogen in vivo.
3.2 Experimental procedures

3.2.1 Cell culture, bacterial strains, growth conditions

Caco-2 intestinal epithelial cells and HT-29 intestinal epithelial cells were obtained from the American Type Culture Collection (ATCC, USA) and grown in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) with 4.5 g/l D-glucose, 1 x non-essential amino acids, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) and 10% fetal bovine serum (Gibco). Cells were seeded at high density in polystyrene T75 cm² culture flasks, 6 well plates (6WP) or 12 well plates (12WP) and used for experiments at confluence 4 days after seeding. Cultured cells were used between passages 6-20. Bacterial strains used in this study are listed in Table 3.1. The EPEC in-frame deletion mutants were generated using the suicide vector pRE112 via sacB-based allelic exchange [242], while the C. rodentium nleC deletion mutant was created using the lambda Red recombinase system [243]. All strains were grown from single colonies on LB plates in LB broth at 37 °C overnight with shaking.
### Table 3.1: EPEC and *Citrobacter rodentium* strains used in this study.

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<td>$\Delta fliC/\Delta escN$ EPEC</td>
<td>T3SS and Flagella</td>
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<td>$\Delta fliC/\Delta nleC$ EPEC</td>
<td>FliC and NleC</td>
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<td>$\Delta fliC/\Delta nleC/nleC$ EPEC</td>
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<tr>
<td>DBS 100 <em>Citrobacter rodentium</em></td>
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*rodentium*

| $\Delta nleC$ *C. rodentium*      | NleC                            | [252]     |
| $\Delta nleC/nleC-2HA$ *C. Rodentium* | NleC                          | This study|
3.2.2 Generation of nleC complemented and nleCHA strains

Complementation of nleC was achieved by chromosomal insertion with Tn7 into EPEC ΔnleC. A chloramphenicol marked Tn7 delivery vector was created by subcloning a SacIfrt-cat-frt, Klenow end-filled, fragment from pFCM1 into the EcoRV site of pUC18R6KT-mini-Tn7T[253], yielding pMAC5, and transformed into DH5αpir. The orientation of the cat cassette was confirmed with EcoRI/NcoI restriction digests. The nleC complementation construct was made by fusing the nleBCD operon promoter to nleC that contained 19 nt upstream of the predicted start site. A 348 bp region containing the nleBCD promoter was PCR amplified with oligonucleotides

nleBCD-f (TCAGAATTCCCAAGCTATATGTTAACTGC) and

nleBCDp-r (GTTTATCCATATTTTCTTCACAAC). A 1033 bp PCR product that contains the nleC gene, with 19 bp upstream of the ATG was amplified with oligonucleotides

nleC19-f (GTTGTGAAGAAAATATGGATAAACCAGGGTATTAGATATAAAACATG) and nleC-r (CGACGGATCCTCCTCATCGCTGATTGTGTTTGTC). The promoter and nleC gene were fused by PCR sewing, using 1 ng of each PCR product as template in a single PCR reaction as previously described [254], using oligonucleotides nleBCD-f and nleC-r. An HA-tagged nleC was also amplified using the same templates and PCR sewing protocol, except using oligonucleotide

nleCHA-r (CGACGGATCCTAAGCGTAATCTGGAACATCGTA TGGGTATCGCTGTTGTGTTTGTC) instead of nleC-r. Both PCR products were sequenced at Nucleic Acid Protein Service Unit (University of British Columbia, Vancouver, British Columbia) and subcloned as an EcoRI/BamHI fragment into a similarly cut pMAC5 yielding pMAC5/19nleC and pMAC5/19nleCHA, and transformed into EC100Dpir. The complementing constructs were mobilized into EPEC ΔnleC by mating with ω7249 [255].
harbouring the Tn7 helper plasmid, pTNS2 [253] and either pMAC5/19nleC or pMAC5/19nleCHA. Transconjugants were selected on LB containing chloramphenicol, and the proper Tn7 insertion was checked as previously described [253]. The resulting complementing strains were designated MCE003 (nleC) and MCE004 (nleC-HA).

3.2.3 Infection protocol
Caco-2 and HT-29 cells cultured in 6 and 12 WP were infected by different strains of EPEC in DMEM Nutrient Mixture F12 (DMEM F-12) for 4 h. At least 30 mins before infection, the medium in 6 or 12 WP was replaced by DMEM F-12 (without supplements). Overnight bacterial culture with an OD$_{600}$ of approximately 0.5 was used for infection. After infection, cells were washed twice with 2ml of PBS and replaced by DMEM growth media containing gentamicin (1 µg/ml) in order to prevent host cell death and the overgrowth of extracellular bacteria. At different time points post-infection, supernatants were collected for sandwich ELISA (24 h) or cell lysates (2-6 h) prepared for Western blotting. For infections using ΔfliC EPEC strains, Caco-2 cells were infected with the bacterial cultures for 3h as described above. Cells were washed with PBS and replaced by 2ml of DMEM F12 media. Purified FliC (100µg) was added for 30 mins to 1 h and cell lysates were prepared for Western blotting.

3.2.4 Measurement of CXCL8/IL-8 secretion levels
The level of CXCL8/IL-8 released into the supernatant was assessed using an ELISA kit (BD Bioscience) following manufacturer’s instructions.
3.2.5 Assessment of bacterial adherence to Caco-2 cells

To assess bacterial adhesion, Caco-2 cells were infected with bacteria for 4 h. Cells were then washed with warm PBS three times and finally scraped into warm PBS and mixed by pipetting. Serial dilutions were performed and aliquots of the scraped cells were streaked on agar plates and incubated in 37°C overnight. Bacterial colonies (CTU) were counted the next day.

3.2.6 NF-κB activity assay

Caco-2 cells were seeded into 24 WP at 2.5 x 10^5 cells/well in 1 mL of media and incubated overnight in a 37°C 5% CO₂ incubator. 1 h prior to transfection, cells were washed with sterile PBS -/- and 0.5 mL of pre-warmed low serum media (0.5% FBS, 1% non-essential amino acids, 1% GlutaMax) was added to each well. Per well, 25ng pNF-κB Luc vector (Clontech Labs) was mixed with 75ng phRL-TK (Renilla) vector (Promega Corporation), and topped up to 500 ng of total DNA with pCMV4a vector (Stratagene). DNA was diluted in 50 µL DMEM and 3 µL of GenJet for Caco-2 cells (SignaGen Laboratories) was diluted in 50 µL DMEM in a separate tube. Diluted GenJet was added directly to diluted DNA and mixed. Transfection mixture was incubated at room temperature for 15 min prior to addition of 100 µL to each well of Caco-2 cells. 24 h post-transfection, media was aspirated from Caco-2 cells and replaced with 0.5 mL pre-warmed serum-free DMEM. Cells were infected with 1 µL of overnight standing EPEC cultures as indicated. Infections were performed in triplicate for each strain used. For un-infected cells, 1 µL of un-innoculated LB media was added to Caco-2 cells. At 2, 3, or 4h post-infection, media was aspirated and replaced with fresh DMEM supplemented with 100µg/mL gentamicin and 5 ng of recombinant human IL-
1β per well for 3 h. The Dual-Luciferase Reporter Assay (Promega Corporation) was used according to manufacturer’s instructions. Briefly, infected Caco-2 cells were washed with 500 µL of sterile PBS -/- and 100 µL of passive lysis buffer was added followed by 15 min incubation at room temperature with rocking. 20 µL of cell lysate was used for luciferase quantification. Data were plotted as Firefly/Renilla to enumerate NF-κB activity relative to a constitutive CMV promoter and NF-κB activity resulting from all infections were plotted relative to uninfected cells, which were set at 100% activity.

3.2.7 Western blotting
Following infection with EPEC, Caco-2 cells were washed twice with 2ml of PBS (Gibco). Cells were then lysed in 100ul of lysis buffer (Promega) on ice for 5-10 min and then scraped into microcentrifuge tubes. The tubes were centrifuged at 13000g for 12 min to pellet debris, and the supernatant was transferred to another tube for Western blots. Caco-2 proteins (50µg in cleared cell lysate) were resolved by 9-11% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2µm polyvinylidene fluoride (PVDF) membranes. Blots were then blocked for 1 h with 5% BSA in Tris-buffered saline with 0.05% Tween-20 (TBST). Membranes were then incubated with primary antibody in TBST overnight at 4 °C and probed with the respective secondary antibody for 1 hr at room temperature. Rabbit polyclonal phospho-p38 MAP Kinase (Cell signalling) and phospho NF-κB p65 (Cell Signalling) antibodies were used in this study.

3.2.8 Mouse infections, cecal loop experiment
Female C57BL/6 mice (8-12 weeks) were purchased from Charles River, and housed at the Child and Family Research Institute. All mouse experiments were performed according to
CCAC guidelines. Cecal loop experiments were adopted from the ileal loop experiments previously described [256]. In brief, mice were anaesthetized by intraperitoneal injection of ketamine and xylazine diluted in sterile PBS. Following a midline abdominal incision, the cecum was exposed and the proximal colon close to the cecum was ligated twice while the ileocecal valve prevented backflow into the ileum, thus isolating the cecum. Bacteria were prepared from overnight cultures in LB and diluted (1:50) in DMEM and left at 37 °C in 5% CO₂ atmosphere for 3 h to pre-activate the bacteria [257]. 300 µl of the pre-activated cultures containing approximately 1 x 10⁷ cfu of C. rodentium were injected into the cecal loop. The intestine was then returned to the abdominal cavity and the incision closed with discontinuous sutures. The mice were euthanized at 12 h and tissues and stool contents were collected for bacterial counts and immunofluorescence.

3.2.9 Tissue collection and bacterial counts
Tissue collection and bacterial counts were performed as described previously [204]. Briefly, mice were euthanized over the course of infection, dissected and their large intestines including the cecum were collected in 10% neutral buffered formalin (Fisher) for histological analyses, or processed for tissue pathology assays. For viable cell counts, colon tissues and stool pellets were collected separately, and homogenized in PBS pH 7.4, with dilutions plated onto LB agar or McConkey agar plates.

3.2.10 RNA extraction, semi-quantitative and quantitative real-time PCR
Following euthanization of mice, colonic tissues were immediately transferred to RNA-later (Qiagen), frozen in liquid N₂ and stored at −20°C. Total RNA was purified using QiagenRNEasy kits (Qiagen) according to the manufacturer's instructions. cDNA was
synthesized with Omniscript RT kit (Qiagen) and OligodT (abm) followed by quantitative real-time PCR techniques. Quantitative PCR was carried out on a Bio-Rad MJ Mini-Opticon Real-Time PCR System (Bio-Rad) using IQ SYBR Green Supermix (Bio-Rad) and MIP-2, MIP3-α and β-actin primers using sequences and conditions previously described (Khan et al, 2006). Quantification was carried out using Gene Ex Macro OM 3.0 software (Bio-Rad) where PCR efficiencies for each of the primer sets were incorporated into the final calculations.

3.2.11 Immunofluorescence staining

Immunofluorescence staining of control and infected tissues was performed using previously described procedures. In brief, tissues were rinsed in ice-cold PBS, embedded in optimal cutting temperature compound (Sakura, Finetech), frozen with isopentane (Sigma) and liquid nitrogen, and stored at −70°C. Serial sections were cut at a thickness of 6 µm, which were directly blocked with endogenous biotin blocking kit (Invitrogen) and 1% bovine serum albumin, followed by the addition of polyclonal rat antisera generated against Tir (1:8000), while the biotinylated anti-HA antibody (Covance) was used at 1:500. Following extensive washing with Tris-buffered saline, Alexa 488 or Alexa 568 conjugated goat anti-rat IgG antibodies or Alexa 488 or Alexa 568 conjugated streptavidin (1:300 dilutions, Molecular Probes) were added. Following extensive washing, the cells were then stained with 1 µg ml⁻¹ of 4’, 6’-diamidino-2-phenylindole (DAPI; Sigma) 1:15 000 for 10 min, washed with PBS before the coverslips were mounted on glass slides in Mowiol (Aldrich) and sealed with nail polish. Sections were viewed at 350, 488, and 568 nm with a Zeiss AxioImager microscope and images were obtained using an AxioCamHRm camera operating through AxioVision software (version 4.4).
3.2.12 **Statistical analysis**
All the results are expressed as the mean value ± standard error of the mean (SEM). Non-parametric Mann–Whiney *t*-tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). A *P* value of 0.05 or less was considered significant.
3.3 Results

3.3.1 Suppression of IL-8 secretion by EPEC is T3SS dependent

Previous studies have indicated that EPEC’s ability to suppress IL-8 secretion by epithelial cells is T3SS dependent \([175,206]\). To confirm this observation, several T3SS mutants were examined: \(\Delta escN\), \(\Delta escF\), \(\Delta escU\), and \(\Delta escV\). Each lack critical components of the T3SS apparatus and is unable to secrete translocator and effector proteins. After infecting Caco-2 cells with WT EPEC for 4 h, followed by antibiotic treatment for a total 24 h of incubation, I recovered 775 ± 70 pg/ml of IL-8. In contrast, all T3SS mutants tested under the same infection conditions induced more than two fold (\(P < 0.05\)) higher of CXCL8/IL-8 levels compared to WT EPEC: \(\Delta escN\) (1591 ± 190 pg/ml), \(\Delta escV\) (1816 ± 203 pg/ml), \(\Delta escU\) (2119 ± 600 pg/ml) (Figure 3.1A). Yet, they were not significantly different from each other which support the hypothesis of the prominent anti-inflammatory role played by EPEC’s T3SS and its effectors.
Figure 3.1: NleC suppresses EPEC induced intestinal epithelial IL-8 responses.

(A) Caco-2 cells were infected with wildtype (WT) EPEC and T3SS EPEC mutants (ΔescN, ΔescF, ΔescU, and ΔescV) for 4 h. Supernatants were collected 24 h later and subjected to IL-8 ELISA. All T3SS mutants induced significantly higher IL-8 release compared to WT infected cells. (B). Caco-2 cells were infected with single gene deletion mutants in the LEE and non-LEE encoded regions as described above. ΔescN, ΔnleC, ΔnleE and ΔnleH mutants induced significantly higher IL-8 release compared to WT infected cells. (C) Complementation of ΔnleC EPEC by chromosomal insertion is seen in Caco-2 cells infected as described above. Complementation of ΔnleC restores the suppressive phenotype. There is no significant differences between WT infected cells compared to cells infected with complemented strain. Error bars for all panels represent the standard error of mean from at least three independent experiments * P < 0.05 One way ANOVA
3.3.2 NleC inhibits IL-8 secretion by EPEC infected cells

Given the T3SS-dependent nature of the inhibition, single gene deletion EPEC mutants within the LEE and non-LEE encoded regions were screened. The LEE-encoded mutants included Δtir, ΔespF, ΔespG, ΔespH, ΔespZ and Δmap, while the non-LEE-encoded mutants screened were ΔnleA, ΔnleB2, ΔnleC, ΔnleD, ΔnleE and ΔnleH1. All mutants contained a functional T3SS and their secretion profiles were normal (aside from the effector of interest - data not shown). Figure 3.1B confirms Nadler’s finding since most mutants were as effective as WT EPEC at inhibiting IL-8 secretion [107]. ΔnleE as well as ΔnleC mutant induced significantly greater levels of IL-8 ($P < 0.05$, $P < 0.01$) compared to WT EPEC infected Caco-2 cells. The ΔnleH1 mutant strain also induced elevated levels of IL-8 ($P < 0.05$), reflecting its suggested ability to block NF-κB function [108].

In previous studies, Dr. Mohammed Khan showed that expression of other pro-inflammatory mediators including β-defensin-2 was also suppressed by EPEC’s T3SS [175]. As expected, I found it was significantly elevated in Caco-2 cells infected with ΔnleC EPEC as compared to WT EPEC infected cells (Figure 3.2). To address whether the exaggerated IL-8 response to ΔnleC EPEC could be seen in another intestinal epithelial cell line, we determined the IL-8 level produced by HT-29 colonic epithelial cells infected with WT, ΔnleC or ΔescN EPEC for 4 h. Similar to Caco-2 cells, ΔnleC EPEC and ΔescN EPEC infected HT-29 produced significantly higher levels of IL-8 (3853 ± 238 pg/ml and 3397 ± 211 pg/ml respectively) compared to cells infected with WT EPEC (2366 ± 206 pg/ml) ($P < 0.05$).
To confirm the action of NleC in the suppression of IL-8 release, we attempted to complement the nleC mutation in EPEC by introducing the nleC gene on the pACYC184 vector (pnlc) to create the ΔnleC/pnlc EPEC strain. Removal of NleC from the EPEC genome did not alter the secretion of translocators or effectors. However, complementation was not achieved by over-expression of NleC, potentially due to changes in the secretion of other effector proteins from EPEC. Since the nleC gene is located in an operon along with nleB, nleH and nleD [252], plasmid based overexpression could alter its transcriptional control in the absence of its normal promoter region, leading to a disruption of the normal functioning of the T3SS, as we recently found with the T3SS effector EspZ [89]. To overcome these issues, chromosomal insertion of the full nleC gene was used to create the ΔnleC/nleC EPEC strain (Figure 3.1C). Lower IL-8 release was seen with Caco-2 cells infected with the ΔnleC/nleC strain (808 ± 50 pg/ml) compared to ΔnleC EPEC infected cells (1181 ± 94 pg/ml) (P = 0.02). The complemented strain produced an IL-8 response of similar magnitude to that seen with WT EPEC (777 ± 70 pg/ml). Similarly, there was no significant difference in the numbers of attached bacteria (WT EPEC: 9.1± 1.1 x10⁶ CFU, ΔnleC EPEC: 8.6 ± 2.2 x10⁶ CFU, ΔnleC/nleC EPEC: 8.0 ± 0.9 x10⁶ CFU) confirming that NleC suppresses IL-8 release in IEC.

### 3.3.3 EPEC NleC inhibits NF-κB activation

NleC has been implicated in the suppression of inflammatory signaling pathways through direct cleavage of the NF-κB p65 subunit [238,239,240,241]. To investigate whether this
signaling pathway was inhibited by EPEC’s NleC in my system, Caco-2 cells were infected for 3h with WT, ΔescN, ΔnleC or ΔnleC/nleC EPEC. NF-κB signaling was examined by Western blot analysis. As expected, NF-κB phosphorylation was inhibited following infection by WT EPEC, as compared to the phosphorylation seen in cells infected by the ΔescN strain (Figure 3.3A). In contrast, NF-κB phosphorylation in cells infected by ΔnleC EPEC was similar to WT EPEC cells. This was surprising but since the ΔnleC EPEC strain still possesses the effectors NleE and NleH1, which may compensate for NleC, perhaps, a stronger NF-κB stimulation was needed to detect differences. To address this, Dr. Stephanie Shames infected Caco-2 cells for 3h with the several EPEC strains, followed by 3 h gentamicin treatment and stimulation with a stronger inducer of NF-κB activation, ie. IL-1β. To specifically examine NF-κB activity, Dr. Shames transfected Caco-2 cells with plasmids containing Firefly and Renilla Luciferase genes under the control of the κB enhancer and CMV promoter, respectively, which provided us with a quantitative readout of NF-κB activity influenced by translocated effector proteins. Data are shown as Firefly/Renilla Luciferase levels and plotted relative to uninfected and IL-1β stimulated cells set to 100% NF-κB activity. As shown in Figure 3.3B, NF-κB activation decreased during WT EPEC infection compared to ΔescN, ΔnleC EPEC infected cells. Yet, NF-κB activation was significantly greater in cells infected with either ΔescN or ΔnleC EPEC when compared to WT EPEC infected cells (P< 0.01 and P< 0.001, respectively) 3 h post infection. Moreover, complementation of the nleC deletion, as assessed by either the ΔnleC/nleC or the ΔnleC/nleCHA EPEC strain, normalized NF-κB activation to WT levels. These findings confirmed the impact of T3SS effector NleC on NF-κB activation during EPEC infection of Caco-2 IEC.
Figure 3: NleC suppresses epithelial inflammatory responses by impairing phosphorylation of NF-κB and p38 MAP kinase.

(A). Caco-2 cells were infected with WT, ΔescN, ΔnleC, ΔnleC/nleC for 3h. Cell infected with bacteria washed in PBS to removed flagellin in the supernatant were marked with + in the Washed column. At the end of the infection cells were lysed and NF-κB p65 subunit phosphorylation were examined through Western blotting. Lane 9 was uninfected control. (B). NF-κB activity assay at 3h post infection using a dual-luciferase reporter system. Caco-2 cells transfected with the constitutively expressed Renilla luciferase gene and Firefly luciferase gene under control of the NF-κB enhancer were infected with the indicated strains or left uninfected. Luciferase activity was quantified post infection and following treatment with IL-1β (see Experimental Procedures). Data are plotted as Firefly/Renilla and 100% NF-κB activity was set for uninfected, IL-1β treated cells. Stars denote statistical significance as compared to NF-κB activity from cells infected with WT EPEC at the same time point * P< 0.01 and ** P< 0.001. Figure 3.3B was prepared by Dr. Stephanie Shames.

3.3.4 NleC localizes to the EPEC induced pedestal.

I next examined the cellular localization of NleC within infected IEC, using a strain of EPEC (ΔnleC/nleCHA) that contained a chromosomal insertion of NleC tagged with two haemagglutinin (HA) epitopes at the C-terminus of the NleC protein. The resulting strain produced high levels of HA-tagged NleC that was secreted in a T3SS-dependent manner. Following standard EPEC infection and effector staining protocols [89], we identified strong HA staining within the EPEC induced pedestal (see Figures 3.4A and B) underlying the adherent EPEC similar to the observation by Yen et al. [241]. In fact, the HA staining partially overlapped with staining for the translocated effector Tir, indicating NleC is
translocated into host cells where it predominantly resides at the tip of the EPEC induced pedestal. The specificity of the staining for HA was confirmed, as no HA staining was detected in cells infected with WT EPEC (lacking HA tagged NleC) (Figure 3.4A and B). Since the localization of NleC near the host cell membrane would not fit with NleC’s role in degrading the p65 subunit of NF-κB, this prompted me to examine whether other inflammatory signaling pathways were also suppressed by NleC.

Figure 3.4: The HA tagged effector NleC localizes to the host cell membrane underlying adherent EPEC. (A) Immunofluorescence staining of HeLa cells infected with either WT EPEC, or ΔnleC- nleCHA EPEC. (B) Inset from Panel A. Bacteria were preactivated with DMEM and cells were infected with either WT or NleC-2HA EPEC for three hours after preactivation. NleC -2HA was stained in red, Tir was stained in green and DAPI was blue. Note in the merge that the HA staining overlaps with the Tir staining, and both are found underlying the adherent ΔnleC- nleCHA EPEC.
3.3.5 EPEC NleC inhibits p38 MAP kinase phosphorylation

Aside from NF-κB, WT EPEC has been shown to also affect inflammatory signaling by inhibiting the phosphorylation of p38 Map kinase [206]. Through infection with Caco-2 cells as described above, our blotting revealed a modest increase in p38 MAP kinase phosphorylation in ΔnleC EPEC infected cells, although densitometry analysis revealed it was not significantly different from WT EPEC (Figure 3.5A). Based on this response, I addressed whether NleC could also be playing a role modulating p38 MAP kinase signaling, as this pathway is known to play an important role in the production of IL-8 in our system. Notably, TLR5 recognition of FliC causes phosphorylation of p38 MAP kinase within 30 mins of stimulation, possibly prior to the actions of T3SS effectors like NleC and thereby limiting our ability to assess the roles of specific T3SS effectors [175]. To overcome this obstacle and better define the signaling pathways altered by NleC, I washed the EPEC cultures before adding them to the epithelial cell cultures. While this would remove shed flagella within the culture, the bacteria would remain flagellated. As shown in Figure 3.5A, I detected a stronger and significant effect of NleC on p38 MAP Kinase signaling in these samples. To further address whether the initial stimulation with FliC was interfering with the function of NleC, I infected Caco-2 cells with EPEC mutants lacking FliC (ΔfliC, ΔfliC/ΔescN, ΔfliC/ΔnleC and ΔfliC/ΔnleC/nleC EPEC). Cells were infected for 3h followed by 30 min of FliC stimulation. After stimulation, cells were lysed and proteins quantified by Western blot followed by densitometric analysis. At 30 min post-FliC stimulation, NF-κB phosphorylation following ΔfliC/Δnle C infection was similar to that seen following ΔfliC infection, suggesting that this approach did not alter general pro-inflammatory signaling (Figure 3.5B). In contrast, both ΔfliC/ΔescN and ΔfliC/ΔnleC EPEC induced significantly (P< 0.05) higher levels of p38 MAP kinase phosphorylation compared
to cells infected with the ∆fliC and ∆fliC/ΔnleC/nleC EPEC (Figure 3.5 C). Densitometry revealed approximately 2 fold higher phosphorylation of p38 MAP Kinase in cells infected with ∆fliC/ΔnleC EPEC as compared to cells infected with ∆fliC EPEC (Figure 3.5C). In contrast, ERK 1/2 and JNK activation were not altered at the same time point (data not shown). Overall, these data suggest that NleC affects p38 MAP kinase signaling in host cells.
Figure 3.5: NleC suppresses epithelial inflammatory responses by impairing phosphorylation of p38 MAP kinase.

(A) Caco-2 cells were infected with WT, ΔescN, ΔnleC, ΔnleC/nleC for 3 h. Cell infected with bacteria washed in PBS to removed flagellin in the supernatant were marked with + in the Washed column. At the end of the infection cells were lysed and p38 MAP Kinase phosphorylation were examined through Western blotting. Lane 9 was uninfected control. At 3 h post infection ΔescN (Washed -) and ΔnleC (Washed -) induced significantly higher p38 MAP Kinase phosphorylation. (B) Caco-2 cells were infected with ΔfliC, ΔfliC/ΔescN, ΔfliC/ΔnleC, ΔfliC/ΔnleC/nleC EPEC for 3 h followed by 30 mins of FliC stimulation, or just received the FliC stimulation (FliC). At the end of the infection, phosphorylation of NF-κB and p38MAP Kinase was probed in the lysate through Western blotting. (C) Densitometric analysis of phosphorylated p38MAP Kinase (p-p38MAP/p38MAP) following infection and stimulation as outlined in (C). ΔfliC/ΔescN and ΔfliC/ΔnleC, as well as FliC stimulation on its own induced significantly higher phosphorylation of p38MAP Kinase compared to ΔfliC and ΔfliC/ΔnleC/nleC infected cells. Stars denote statistical significance of *P< 0.05
3.3.6  *C. rodentium* ΔnleC shows no defects in colonization but causes exaggerated colitis

Based on our observation that EPEC NleC plays an important role in suppressing epithelial inflammatory responses *in vitro*, we next tested whether NleC expressed by *C. rodentium* had similar effects *in vivo*. I infected C57BL/6 mice with either WT or ΔnleC *C. rodentium*, and enumerated the pathogen burdens found within the ceca and colons of infected mice at day D6 and D10. Interestingly, *C. rodentium* numbers were similar between the two groups, both in the cecum and the colon at both time points (cecum: D6- WT: $2.0 \pm 1.7 \times 10^7$ CFU/g, ΔnleC: $4.3 \pm 1.4 \times 10^6$ CFU/g, D10- WT: $6.6 \pm 3.6 \times 10^8$ CFU/g, ΔnleC: $9.1 \pm 5.2 \times 10^8$ CFU/g; colon: D6- WT: $6.3 \pm 1.8 \times 10^8$ CFU/g, ΔnleC: $2.9 \pm 0.9 \times 10^8$ CFU/g, D10- WT: $5.2 \pm 1.7 \times 10^8$ CFU/g, ΔnleC: $1.8 \pm 0.6 \times 10^8$ CFU/g) (see Figure 3.6A). I also assessed the tissue pathology in these mice, blindly scoring histological sections for edema, goblet cell depletion, hyperplasia and tissue integrity as well as inflammatory cell infiltration as previously described [200]. I found that at D6, the mean histological score for the ΔnleC *C. rodentium* infected ceca was $2.58 \pm 0.20$, a level significantly higher than the score of $0.68 \pm 0.28$ obtained for WT *C. rodentium* infected cecal tissues (Figure 3.6B). The ΔnleC score reflected worsened edema, hyperplasia, and increased inflammatory cell infiltration. At D10, the elevated pathology scores were maintained in the ceca of ΔnleC *C. rodentium* infected mice, at $2.60 \pm 0.40$, a level still significantly higher than the score of $1.10 \pm 0.40$ obtained for WT *C. rodentium* infected cecal tissues (Figure 3.6B). Specifically, at D10 we observed more inflammatory cell infiltration and goblet cell depletion in the ΔnleC *C. rodentium* infected cecal tissues. While the scores for the colons of the ΔnleC *C. rodentium* infected mice were also elevated at both D6 and D10, they did not reach statistical significance (Figure 3.6C). As bacterial colonization did not differ between the infection groups at either
D6 or D10 p.i., the increased damage suffered following ΔnleC C. rodentium infection appears to reflect the actions of NleC.

**Figure 3.6: Impact of NleC on C. rodentium colonization and colitis.** C57BL/6 mice were infected with WT or ΔnleC C. rodentium for six and ten days.

Pathogen burdens were enumerated and there were no significant differences in bacterial colonization in the cecum and colon at these time points. (B). Histological scoring analysis of H&E stained tissues revealed worsened histology scores in the ceca of mice infected with ΔnleC C. rodentium at both time points.

### 3.3.7 C. rodentium ΔnleC outcompetes WT C. rodentium in competitive index studies

Considering the impact of NleC in limiting CXCL8/IL-8 release *in vitro* as well as its role in controlling host pathology *in vivo*, we found it surprising that ΔnleC C. rodentium showed no defects in colonization levels when compared to WT C. rodentium. However, comparing colonization levels in separate hosts is a relatively insensitive measurement of virulence. In
fact, previous studies using this approach found only modest colonization defects in *C. rodentium* strains lacking NleE or NleH [108,238,258]. This may reflect the fact that *C. rodentium* is a highly adapted murine pathogen, and can readily colonize mice, even when it is lacking important virulence factors. Therefore, I tested the Δ*nleC* mutant using the competitive index (CI) assay, a more sensitive measure of virulence that we have repeatedly used in the past. In a CI assay, the same animal is infected with a culture containing both WT and mutant strain at a known ratio. At the end of the experiment, the ratio between the WT and mutant strain will be reevaluated. If the mutant strain does not suffer from any deficiency in virulence, the CI value will remain as 1. If the WT strain is more competitive/virulent than the mutant tested, CI value will be less than 1. In contrast, if the mutant strain is more competitive/virulent than the WT strain, the CI value will be greater than 1. After infecting C57BL/6 mice with equal numbers of WT and Δ*nleC* *C. rodentium*, and collecting tissues at D4, I found the Δ*nleC* strain to have a surprisingly high CI of 4.41 ± 0.98 (Figure 3.7A), indicating that the mutant strain significantly outcompeted WT *C. rodentium* in colonizing the murine GI tract. The determination that *C. rodentium* Δ*nleC* can out-compete WT *C. rodentium* when co-infected *in vivo* was highly reproducible and interestingly, the competitive advantage displayed by the Δ*nleC* strain was maintained, albeit at a slightly lower level at D7 (Figure 3.7A). To better define the basis for the high CI, in additional mice we separated the luminal stool contents from the gut tissues, and I collected the stool as well as the cecal and colonic tissues separately. When the CI was calculated for these different isolates, the CI for tissue adherent Δ*nleC* *C. rodentium* in the cecum was 1.06 ± 0.19. In contrast, the CI in the stool was 2.68 ± 0.91 while the CI in the distal colon was 9.54 ± 4.06 (Figure 3.7B). Overall these data show that Δ*nleC* *C. rodentium* colonizes the cecum at
roughly equal efficiency with the WT strain, but the mutant outcompetes WT *C. rodentium* in spreading down the GI tract to colonize the distal colon.

Figure 3.7: ∆nleC *C. rodentium* outcompeted WT *C. rodentium* in vivo

(A) C57BL/6 mice were infected with WT *C. rodentium* and ∆nleC *C. rodentium* for four and seven days and cecal plus colonic tissues were collected for bacterial enumeration. At both time points, ∆nleC *C. rodentium* outcompeted WT *C. rodentium* with a competitive index of 4.41 ±0.98 (P< 0.05) and 2.13 ± 0.88 respectively. The dotted line indicates a CI of 1. Error bars indicate the standard error of mean values obtained independently from at least 6 mice. (B) CI for luminal content, cecum and colon at D4 post infection. The CI for tissue adherent ∆nleC *C. rodentium* in the cecum (1.06 ± 0.19), stool (2.68 ± 0.91) and distal colon (9.54 ± 4.06).

3.3.8 Cecal loop infection shows NleC translocates into IEC in vivo

The increase in inflammatory cell infiltration and tissue damage seen in the intestines of mice infected by ∆nleC *C. rodentium* (alone) at D6 and D10 demonstrates that NleC modulates inflammatory responses *in vivo*, however the severity of the resulting colitis was less than expected, compared to the dramatic effect that NleC plays in suppressing IL-8 release from IEC. In part, the impact of NleC on the colitic response may have been mitigated by the involvement of inflammatory responses from uninfected IEC, as well as macrophages and other cell types that were not directly infected, but instead were stimulated by shed bacterial products that activate innate receptors including TLR2 and TLR4 [203,208,259 ]. To more
accurately assess the impact of NleC or other T3SS effectors on epithelial derived inflammatory responses *in vivo*, I hypothesized that the analysis needed to occur very early during infection, to limit the contribution of uninfected IEC and other cells. Unfortunately, very few *C. rodentium* initially colonize the murine GI tract following oral gavage, with heavy colonization of the epithelium not occurring until D4 or D6 [112], too late for my purposes. To overcome this problem, I utilized a recently developed cecal loop model, by which we inject a large number of pre-activated *C. rodentium* into the cecum. *C. rodentium* were incubated with DMEM for 3 h to induce type 3 secretion [87]. This approach allows us to synchronize their arrival and subsequent direct infection of large regions of the cecal epithelium.

To test the efficacy of the model, I first assessed whether NleC could be detected within the IEC of mice following infection of the cecal loop. Dr. Mathew Croxen generated a plasmid expressing NleC tagged with two HA epitopes at the C-terminus of NleC protein. After incorporating this construct into ∆nleC *C. rodentium*, the resulting strain (∆nleC/nleC-2HA) produced high levels of HA-tagged NleC that was secreted in a T3SS dependent manner. I infected cecal loops with ∆nleC/nleC-2HA *C. rodentium* or WT *C. rodentium* and after 12 h, the tissues were collected and stained for HA, as well as the translocated effector Tir, to identify the sites of *C. rodentium* attachment to host cells (Figure 3.8A). Tir staining was seen on the apical surface of infected epithelial cells, lying beneath the adherent bacteria (Figure 3.8B). Similar to our *in vitro* assessment, the HA signal was found to overlap with Tir on the surface of cells infected with ∆nleC/nleC-2HA *C. rodentium*. Notably, HA
staining was not seen in uninfected (Tir negative) cells, or in IEC infected by WT C. *rodentium* (Figure 3.8A to B).

Figure 3.8: *Cecal loop model permits observation of NleC translocated into IEC in vivo.* Ceca of C57BL/6 mice were surgically ligated and injected with pre-activated Δ*nleC/nleC-2HA* *C. rodentium* for 12 h. At the end of the infection, ceca were collected for immunostaining. (A) Δ*nleC/nleC-2HA* *C. rodentium* infected cecal epithelium (original mag 1000x). (B) Inset from panel A showing that HA signal (green) overlaps with Tir (red) on the surface of cells infected with Δ*nleC/nleC-2HA* *C. rodentium*. Nuclei of cells and bacteria were stained with DAPI (blue). The overlap of the signal indicated NleC-HA protein is localized near the site of pedestal formation (Tir).
3.3.9 **C. rodentium ΔnleC infection induces significantly higher chemokine expression**

Since the cecal loop model permitted effective and rapid infection of the cecal epithelium, with subsequent translocation of *C. rodentium* effectors into infected host cells, I next used this model to compare the inflammatory responses elicited by WT and ΔnleC *C. rodentium*. 12 h following injection, cecal loops were dissected, and tissue samples were homogenized and plated to enumerate *C. rodentium* while other samples were collected for RNA extraction. *C. rodentium* colonization in the cecum did not differ significantly between mice infected with WT (7.5± 2.9 x 10⁷ CFU/g) and ΔnleC *C. rodentium* (8.7± 4.6 x 10⁷ CFU/g). After isolating mRNA, quantitative RT-PCR was performed to examine the expression of *MIP-2, MIP-3α and KC*. All three genes encode chemokines that are expressed by IEC and can putatively attract macrophages, dendritic cells and/or neutrophils to the site of infection. In comparison to cecal tissues taken from mice infected with WT *C. rodentium*, cecal tissues taken from mice infected with ΔnleC *C. rodentium* expressed significantly higher mRNA levels (*P* < 0.05) of *MIP-2* (5.2 fold), *MIP-3α* (2.9 fold) and *KC* (5.8 fold) compared to mice infected with WT *C. rodentium* (Figure 3.9), confirming that ΔnleC *C. rodentium* triggers a significantly greater inflammatory response than WT *C. rodentium*. 
Figure 3.9: Impact of NleC on bacterial colonization and inflammatory cytokine production in the cecal loop model.

(A) qPCR was performed to examine the gene expression of the chemokines MIP-2, MIP-3α and KC. Tissues infected with ΔnleC C. rodentium expressed significantly higher mRNA levels of MIP-2, MIP-3α and KC compared to mice infected with WT C. rodentium. Error bars represent the standard error of mean from at least three independent experiments. * P<0.05. One way ANOVA with Boneferri Post test.
3.4 Discussion
Following their ingestion, enteric bacterial pathogens must not only survive the harsh environment of the host’s GI tract but also avoid or overcome an array of mucosal defenses in order to replicate and successfully colonize their hosts. Unlike the pathogens *Shigella* and *Salmonella* that invade the intestinal epithelium, thereby escaping the gut and its luminal defenses, the diarrheagenic pathogens EPEC and EHEC colonize the mucosal surface by attaching to the apical surface of epithelial cells. How these pathogens remain at the mucosal surface, surviving and multiplying within the luminal environment, while being exposed to all the anti-microbial responses elicited by the underlying epithelial cells remains unknown. However, recent studies have found that, aside from subverting the host cell cytoskeleton, and modulating host cell apoptosis, A/E pathogens also suppress inflammatory signaling, presumably as a means to create a protected niche on the surface of the infected epithelium.

This virulence strategy has been demonstrated through several studies showing that WT EPEC infection suppresses the expression of the chemokine CXCL8/IL-8 and several other epithelial derived mediators including MIP-3α and β-defensin-2. Moreover, this suppression was shown to occur in a T3SS dependent manner, and was linked to the inhibition of several signaling pathways including NF-κB and p38 MAP Kinase. Similarly, in an earlier study examining IEC responses during *C. rodentium* infection in mice, we found that expression of the inducible nitric oxide synthase (iNOS) enzyme was dramatically upregulated in the colonic epithelium during later stages of *C. rodentium* infection [202]. Interestingly, further examination revealed that the iNOS expression was almost solely localized to uninfected IEC, whereas infected cells showed little if any expression of this pro-inflammatory enzyme. Since *C. rodentium* lacking a T3SS are unable to colonize the intestines of mice, we were
unable to address whether the inhibition of iNOS was T3SS dependent, however these findings indicate that A/E pathogens do subvert epithelial inflammatory responses in vivo.

Until recently, the T3SS dependent effectors that mediate this suppression have remained undefined; in this study, I confirmed the T3SS effector NleC plays an important role in suppressing IL-8 release from infected IEC. NleC is encoded on the same operon as the effectors NleG, NleB, NleH and NleD, all of which are found on the PP4 lambda-like prophage in the EPEC genome [86]. A 330 amino acid protein with a molecular mass of 36 kDa [252], NleC is a homolog of the apoptosis inducing effector AIP56, encoded by Photobacterium damsela [260,261]. EPEC’s NleC shares 100% and 95% identity with NleC from EHEC and C. rodentium respectively, suggesting the effector’s function is likely conserved among A/E pathogens. Modulation of inflammatory signaling is the first role attributed to NleC, as it was not found to be involved in either A/E lesion formation or adherence [252].

We and others have previously shown that EPEC induces IL-8 expression in IEC through MAPK and NF-κB activation in response to TLR5 dependent recognition of FliC. In this study, my data suggested that NleC interferes with both NF-κB and p38 MAPK signaling, resulting in heightened IL-8 release from infected IEC. Interestingly, while the impact of NleC on IL-8 release was dramatic, its effects on the pro-inflammatory signaling pathways were subtle. I was unable to detect an overt difference in NF-κB activation following infection by the ∆nleC EPEC strain alone, but only following stimulation by IL-1β. Similarly, Baruch et al recently demonstrated that NleC suppresses NF-κB activation in IEC.
in response to TNF-α induced IL-8 secretion, however they did not examine whether NleC impacted on MAP kinase activation, perhaps because MAP kinases do not play a major role in TNF-α induced signaling [238]. In contrast, I detected significantly elevated p38 MAPK phosphorylation following ΔnleC EPEC infection, but only by delaying FliC stimulation until 3 h post infection. The subtle nature of these changes may reflect the actions of other T3SS effectors playing compensatory roles, as well as the nature of the assays and the pro-inflammatory stimuli used. I also showed for the first time that NleC impacts on intestinal inflammatory responses \textit{in vivo}. While little is known about the role of NF-κB in determining the contribution of IEC to \textit{C. rodentium} colitis, we and others recently showed that IEC specific p38 MAP kinase signaling plays a major role in the immune and inflammatory response to \textit{C. rodentium} infection [175,206,262], in keeping with our findings that the ΔnleC strain triggered greater chemokine responses \textit{in vivo}. It has been reported recently that p38Map kinase modulates NF-κB signaling during \textit{C. rodentium} infection [263] and these signaling pathways are important in MIP2, MIP-3α and KC production in epithelial cells [264,265,266]. By suppressing both NF-κB and p38MAP kinase activation, \textit{C. rodentium} may lower the local inflammatory response by modulating the release of inflammatory chemokines through injected effectors such as NleC.

Previously, NleC was shown to translocate into host cells but its localization within the host cell remains controversial [252]. Baruch \textit{et al.}, recently showed ectopically expressed NleC localizing to the host cell nucleus [238] whereas Yen \textit{et al.}, showed NleC localized near the pedestal [241]. Through the use of HA tags, our data align with the observations made by Yen \textit{et a.l} [241], as I localized NleC primarily to the sites of pathogen attachment, both \textit{in
vitro and in vivo. This was seen in both EPEC and C. rodentium induced pedestals. The staining of the HA tag partially overlapped with staining for the T3SS effector Tir, potentially indicating that it is found at the apical cell membrane, just beneath the bacterium, although it is certainly possible that NleC ultimately reaches other sites in the host cell, including the nucleus, where it may be too diffuse to detect by immunofluorescence. While the binding partner of NleC has not yet been identified, recent studies have indicated NleC functions as a metalloprotease, and can cleave p65 [238], potentially explaining how it impacts NF-κB function, however our findings that bacterially delivered NleC impacts MAP kinase signaling and predominantly localizes to the pedestal suggests it may have other roles, potentially at the host membrane. By localizing near the host membrane, it may allow NleC to suppress induction of inflammatory response; therefore, affecting downstream signaling molecules such as p38 MAP kinase and NF-κB. Interestingly, despite having suppressive effect on both p38MAP kinase and NF-κB signaling, NleC does not suppress ERK 1/2 activation demonstrating the specificity of the suppressive effect.

Over the last two years, other EPEC T3SS dependent effectors have been identified as suppressing IL-8 release and modulating NF-κB activation in epithelial cells. NleE and NleB were shown to bind to NF-κB and in its absence NF-κB signaling in HeLa cells was increased 20 fold. This was associated with increased levels of IL-8 mRNA in infected HeLa cells [106,107]. Similarly, NleH1 has been reported to bind ribosomal protein S3 to subvert NF-κB function and infection by an nleH1 mutant induced higher levels of IL-8 gene transcription than WT EPEC [108]. Curiously, none of the effectors was tested using epithelial cells of intestinal origin, therefore, I tested EPEC strains lacking these effectors and
confirmed both NleE and NleH1 suppress IL-8 secretion in IEC lines, in concert with NleC. Interestingly, the NleB mutant does not induce a higher IL-8 release in infected cells. As reported previously, NleE expression but not NleB expression partially restored EPEC’s capacity to stabilize IκB [107]. In the NleB mutant where NleE is presence, NleE is sufficient to suppress NF-κB to prevent induction of IL-8 release. Taken together, it appears that A/E pathogens use at least these four effectors to inhibit inflammatory responses by suppressing both NF-κB and p38MAPK signaling pathways.

In keeping with its immunosuppressive role in vitro, NleC also inhibited C. rodentium induced colitis following oral gavage. However this route of infection is not an ideal system to address C.rodentium effector functions as it has frequently proven difficult to show significant attenuation in colonic pathology, even with bacterial strains lacking important virulence factors. We believe this lack of concordance between in vitro and in vivo actions reflects the initially low pathogen burden seen during the first 4 days of C. rodentium infection, resulting in very little intestinal inflammation. By 4 to 6 days post-infection when the infection has widely spread throughout the lower bowel and inflammation is evident, we estimate at most, only 20% of IEC are actually directly infected and presumably A/E pathogens can only suppress the responses of cells they directly infect. Moreover, the resulting inflammatory responses seen during these infections are not solely arising from the infected epithelium, but also from uninfected IEC and immune cells exposed to shed bacterial products such as flagellin. These complications likely explain why NleC was found to play only a modest role in modulating the colitis caused by C. rodentium infection, yet had a striking effect on chemokine responses in a cecal loop model, where I maximized IEC
infection, prior to the recruitment of significant numbers of inflammatory cells. Our in vivo results also raise a question concerning our in vitro findings that over a 4 h time course, NleC was unable to block flagellin induced epithelial p38Map kinase signaling that occurred prior to the injection of NleC. This finding is likely of little relevance to an in vivo setting, where the epithelium targeted by A/E pathogens is covered by a mucus layer that would limit exposure to shed bacterial products prior to direct infection. Moreover, in an in vivo setting, A/E pathogen infection of specific epithelial cells could possibly last for days, meaning that translocated effectors like NleC would be present and presumably abrogating inflammatory signaling for hours or days after the initial infection.

While I demonstrated the impact of NleC on host inflammatory responses and pathology, curiously, I saw no significant attenuation in the ability of the ΔnleC strain to colonize the colonic epithelium of murine hosts when given as a single infection. This may not be surprising, since comparing single strain infections is not a highly sensitive measure of virulence, as C. rodentium is a highly successful pathogen with a multitude of compensatory virulence strategies. Strikingly however, we did identify a robust and significant CI of 4.4 for the mutant, indicating that the ΔnleC strain outcompeted the WT strain by more than 4 fold during the infection. This advantage was most evident at D4 PI, with the advantage modestly reduced by D7 of the infection. The basis for this advantage is unclear, but host inflammatory responses have been previously shown to aid C. rodentium, as well as other enteric pathogens including S. Typhimurium, in their colonization of the murine lower bowel [64,226]. Since C. rodentium appears to initially colonize the intestine in a clonal fashion, localized differences in inflammation and pathologic changes in the gut mucosa could provide the
mutant with a competitive advantage over the WT strain, promoting its spread through the host’s intestines. Alternatively, aside from inflammation, NF-κB and p38MAP kinase dependent signaling also affects other host processes such as cell death and tissue repair. Localized changes in epithelial cell sloughing, for example, could also prove selectively beneficial to the mutant strain. In contrast, previous studies have shown that C. rodentium mutants lacking either of the other two effectors (ie. NleE and NleH1) are outcompeted by WT C. rodentium [104,109]. It is unclear why such divergent competitiveness is seen between ΔnleC, ΔnleE and ΔnleH mutants, since they all cause exaggerated release of IL-8 in vitro. However it may reflect that NleE and NleH1 primarily target NF-κB signaling, whereas NleC also targets p38 MAP kinase. Aside from affecting inflammation, NF-κB also plays an anti-apoptotic role in the intestinal epithelium, therefore while NleC, NleE and NleH may all suppress inflammatory responses, the loss of NleE or NleH may limit C. rodentium’s ability to subvert other key functions of epithelial cells, such as apoptosis, which could significantly impair their competitive fitness. Taken together, this study suggests that NleC is a unique virulence factor that impacts on several aspects of inflammatory signaling. Additional studies are needed to address the probably multivalent actions of NleC, and how it and other T3SS effector work together to modulate host inflammatory responses.
Chapter: Discussion

4.1 Revisiting the role of IEC in mucosal homeostasis

The roles played by IEC within the intestine are highly complex. IEC are not passive cells that act as a simple physical barrier between the host and the commensal microbes residing within the intestinal lumen, but instead, their actions are highly regulated. The innate responsiveness of IEC is suppressed under homeostatic conditions, in order to limit inappropriate responses to commensal microbes, yet IEC can also respond quite strongly once they encounter a bacterial pathogen [24,35,38]. Aside from being hypo-responsive to bacterial products, the intestinal epithelium tolerates the presence of the luminal commensal microbiota through several other approaches, including the release of mucus, and by apically secreting anti-microbial peptides, creating a microbe-free zone above the IEC, and thereby limiting direct exposure to the commensal microbiota [12,25,40]. Maintaining tolerance to commensal microbes is essential to intestinal health as it is clear that developing immune/inflammatory response to commensal bacteria can lead to uncontrolled inflammation and intestinal tissue damage as seen in patients with IBD [17].

While clearly chronic inflammation is detrimental, inflammation is also a necessary part of the host response to remove enteric pathogens. Inflammation also triggers the healing process following the clearance of an infection. Most studies have focused on the role of immune cells residing in the lamina propria in inducing intestinal inflammation [267,268]. In contrast, the inflammatory responses elicited by IEC are poorly understood. Residing at the mucosal interface with the luminal environment, and expressing innate receptors such as TLRs, IEC are well suited to respond to invading pathogens. Indeed, IEC have been shown to release anti-microbial peptides such as Reg3-γ and β-defensin and to activate immune cells by
releasing chemokines and cytokines to co-ordinate the removal of invading microbes [9,10,23,25]. Despite these active responses, compared to professional inflammatory cells, IEC are generally hypo-responsive to most bacterial ligands such as LPS.

By using the Cre-loxP system to delete MyD88 in the IEC of C57BL/6 mice, my findings in Chapter 2 align with other recent findings that MyD88 signalling within IEC does not play a significant role in driving the colitic response against *C. rodentium* or other forms of experimental colitis [179,215,216]. Together, these findings raised the question of why innate signaling in IEC does not play a larger role in these models. When we studied the impact of the negative regulator SIGIRR during *C. rodentium* induced colitis, it became evident that IEC are capable of inducing inflammation against invading microbes, but the magnitude of their response was limited by SIGIRR. Indeed, *Sigirr*⁻/⁻ IEC exhibit much greater inflammatory and proliferative responses at the expense of causing more severe mucosal damage and causing the rapid removal of gut commensal microbes, thereby reducing colonization resistance against bacterial pathogens. The work outlined in this thesis provides the first evidence that one of the benefits of limiting the innate responsiveness of IEC is the promotion of host mutualism with the commensal microbiota. By competing for resources such as nutrients and space, the commensal microbiota limits colonization by pathogenic microbes, thereby protecting the host [121,123]. I think it is likely to be more beneficial to the host to maintain its mutualism with its gut microbiota, thereby preventing many infections, rather than developing a stronger host response once an infection has occurred.
Interestingly, the suppression of IEC inflammatory responses is not solely an approach used by the host. Once a bacterial infection does occur, IEC respond strongly, and many of the IEC mediators they release are highly anti-microbial, or are effective at recruiting inflammatory cells to the site of infection [1,14,24,25]. As a result, these mediators can ultimately eliminate invading pathogens that have successfully bypassed the commensal microbiota and the mucus layer. Therefore, successful pathogens have had to develop virulence strategies that allow them to subvert or prevent the host epithelium from developing a maximally effective anti-microbial response [269]. Interestingly, many bacterial pathogens inject effector proteins into host cells in order to modulate any number of host cell processes, including their inflammatory responses. Through my studies conducted in Chapter 3, I demonstrated that EPEC and C. rodentium both suppress IEC inflammatory responses using the effector protein NleC. NleC limits anti-microbial /inflammatory responses by IEC during both in vitro and in vivo infections. Taken together, my thesis research has advanced our understanding of the central role played by IEC in controlling host responses during enteric infections, and how both the host and the pathogen modulate these responses for their own benefit.

4.1.1 The “active” roles of IEC

Studies have shown that IEC plays a central role in maintaining the physiological steady-state of the mucosa [13,35,38,54]. This role includes maintaining barrier function, releasing baseline levels of antimicrobial peptides and modulating the function of underlying immune cells. While it is clear IEC play a prominent role in all these dimensions, IEC have been generally shown to be hypo-responsive to bacterial ligands under physiological condition. Therefore, to investigate the impact of enhancing IEC responses towards enteric pathogens, I
studied the negative regulator of TLR/IL-1R innate signalling, SIGIRR. Recent studies performed by our laboratory and others have demonstrated that the innate hypo-
responsiveness of IEC reflects, at least in part, the effects of SIGIRR [192,195]. SIGIRR has been shown to dampen IEC responses to bacterial ligands in vitro, and similarly, Sigirr -/- mice exhibited a higher inflammatory tone within their intestines, and were found to be more susceptible to chemical induced colitis. Under baseline conditions, Sigirr -/- mice do not develop spontaneous colitis in spite of their heightened intestinal inflammatory tone. Thus, the interactions between IEC and commensals, at least under baseline conditions, does not lead to any overt aberrant reaction in the Sigirr -/- mice.

In Chapter 2, I conducted experiments to examine whether SIGIRR modulates host responses during C. rodentium infection. Not surprisingly, infected Sigirr -/- mice exhibited heightened inflammatory responses including elevated mRNA levels for genes encoding antimicrobial peptides, as well as pro-inflammatory chemokines and cytokines. Although infected Sigirr -/- mice harboured increased pathogen burdens, they ultimately cleared the infection, and showed no evidence of increased morbidity or mortality. While the exaggerated responses appeared to be protective, they also led to epithelial erosions and mucosal ulcerations, predominantly in the cecum. Remarkably, the elevated inflammatory signalling within the Sigirr -/- mice also enhanced IEC proliferation and repair that protected the mice during infection. In fact, I noted that the loss of SIGIRR was able to compensate for other innate signalling defects. Specifically we have previously shown that TLR2 deficiency causes a significant increase in intestinal permeability (ie. IEC barrier dysfunction) following C. rodentium infection, in concert with a reduction in STAT3 phosphorylation [208]. In
contrast, I found that infected *Sigirr/Tlr2* -/- mice were protected from exaggerated barrier dysfunction, and showed strong pSTAT3 staining in crypt IEC.

Further examination revealed that the exaggerated IEC proliferative and barrier responses seen in *Sigirr* -/- mice were dependent on IL-1R signalling as *Il-1r/Sigirr* -/- mice did not display these protective IEC responses during *C. rodentium* infection. The loss of SIGIRR improved mucosal barrier function and prevented *C. rodentium* from spreading systemically. Through BM transplantation studies, I found that the cellular source of SIGIRR governing the augmented healing and innate responses was non-hematopoietic cells (putative IEC). By isolating crypts from the *Sigirr* -/- mice, I found they possessed greater bactericidal ability against commensal microbes than crypts from wildtype mice. It thus appears that SIGIRR deficiency leads to more active IEC, both in terms of proliferation, inflammation and antimicrobial responses. Finally, although I did not look at this in great detail, SIGIRR deficiency is also known to promote the development of T helper (Th)17 cells [194]. Th17 cells produce IL-17A which is known to play a protective role during *C. rodentium* infection [117]. Aligning with this result, I observed higher transcript levels of IL-17A in the *Sigirr* -/- mice following infection. The increased induction of Th17 responses presumably assists in the clearance of *C. rodentium* in *Sigirr* -/- mice. My data thus suggest that IEC, in the absence of negative regulators like SIGIRR, may provide the host with an enhanced capability to fend off infections by leading to increased antimicrobial responses, stronger barrier function and elevated adaptive immune responses. With all these benefits, it raises the question of why mammalian IEC have evolved to maintain their innately hypo-responsive state?
4.1.2 Exploring the benefits of hypo-responsive IEC

While it appears that increasing the innate responsiveness of IEC can provide the host with a number of benefits, not surprisingly, there are also several potential drawbacks. The experiments conducted in Chapter 2 of this thesis demonstrate that having hyper-responsive IEC leads to increased intestinal pathology, as well as a reduction in colonization resistance against enteric pathogens. The exaggerated mucosal inflammatory pathology suffered by the Sigirr -/- mice, including ulcerations were ultimately repaired, via increased IEC proliferation but we also know that such rapid proliferation can elevate the chances for mutations and for tumorigenesis [198,270]. As expected, Sigirr -/- mice are more susceptible to cancer development in a number of relevant cancer models, in terms of tumour size and numbers. In contrast, transgene expression of SIGIRR by IEC alone, rescues Sigirr -/- mice from their increased susceptibility to colon cancer [195,198,271]. Thus, suppressing inflammatory IEC responses by the negative regulator SIGIRR lower the risks of tumorigenesis. Increased innate responsiveness by IEC at the mucosal surface can also increase the risk of other inflammatory GI diseases, including Crohn’s disease and ulcerative colitis. Studies have shown that inflamed mucosal regions from the colons of ulcerative colitis patients express significantly lower levels of SIGIRR compared to non-inflamed regions [193]. Taken together, these results suggest IEC responses are normally suppressed by SIGIRR, to down-regulate inflammatory responses and prevent tumorigenesis and other inflammatory GI diseases (Figure 4.1).

It is often assumed that the innate hypo RESPONSIVENESS of IEC is designed to prevent excessive inflammation and tissue damage in the intestine in response to commensal
microbes. In fact, repeated studies have shown that innate responses to commensal microbiota worsens the severity of most chemical models of experimental colitis, while commensal microbes do appear to help drive the chronicity of the inflammation in IBD patients [12,13,195,209]. My work in Chapter 2 of this thesis provides another explanation for why IEC are innately hypo-responsive. I clearly showed that Sigirr -/- mice display increased susceptibility to infection by several bacterial pathogens. Their vulnerability to infection was associated with a rapid depletion of commensal microbiota from the intestinal lumen, suggesting the anti-microbial response observed in the Sigirr -/- mice induced the rapid removal of the commensal microbiota instead of preventing C. rodentium colonization. I propose that this removal of the commensal microbiota reduced competition for the invading microbes, lowering the infectious threshold required for the pathogens to colonize (Figure 4.1). Thus, by having innately hypo-responsive IEC, the host is better able to tolerate the presence of commensal microbes, thereby reducing the risk of pathogen colonization. Therefore my work on Sigirr-/- mice in Chapter 2 advances our knowledge of the mechanisms that promote intestinal mutualism with commensal microbes and prevent colonization by invading pathogens. This ingenious host defense mechanism likely developed in concert with the other benefits to the host provided by the gut microbiota, such as the large repertoire of digestive enzymes that are not encoded by the host genome. The commensal microbes thus contribute to the digestion of dietary substances and the synthesis of essential food supplements such as vitamins [4]. Furthermore, the presence of the commensal microbes within the intestine is critical to the development and maturation of the mucosal immune system [12]. Finally, as I have outlined in the above section, commensal
microbes provide protection from infection by competing with potential pathogens for space and nutrients, with this protection termed “colonization resistance”.
Figure 4.1: Proposed mechanism for SIGIRR function

The main role for SIGIRR in the intestine is to act as a regulator for IEC response. SIGIRR regulate the innate immune responsiveness of IEC to commensal microbes at steady state to reduce susceptibility to enteric infections (left). During enteric infection, SIGIRR expression modulate IEC response enables a controlled and effective reaction against enteric infection without the excessive tissue damage.
4.1.3 Colonization resistance

The concept of colonization resistance is not novel and in fact, numerous studies have shown that commensal microbes play a key role in controlling host susceptibility to infection by enteric bacterial pathogens [68,121,123,272]. For example, germfree mice lacking commensal microbes are readily infected by the pathogens *S. Typhimurium* and *C. rodentium*, and typically suffer more severe infection induced mucosal injuries compared to commensal carrying mice [126,221]. While germfree mice are essentially lacking intestinal colonization resistance, studies have also shown that even a partial decrease in the numbers/makeup of commensal microbes through antibiotic treatment can dramatically reduce colonization resistance. Antibiotic treatment decreases the numbers of microbes as well as the species diversity within the intestine, and thereby reduces colonization resistance by opening up niches and nutrients for invading pathogens to exploit [210,221]. In fact, the current gold standard for modelling *S. Typhimurium* induced colitis depends on the pretreatment of mice with the antibiotic streptomycin to reduce commensal competition with orally gavaged *S. Typhimurium* [126]. Normally, *S. Typhimurium* can only poorly colonize the mouse intestine, but by reducing and shifting the makeup of the commensal microbiota, streptomycin pretreatment allows *S. Typhimurium* to readily colonize the murine cecum and colon. Notably, we found that *S. Typhimurium* colonized and induced cecitis/colitis in *Sigirr* -/- mice without the typical oral treatment by antibiotics. As far as I am aware, this is the first mouse strain described to develop such heavy *S. Typhimurium* colonization of the GI tract, without streptomycin pretreatment. This susceptibility to *S. Typhimurium* colonization clearly demonstrates that *Sigirr* -/- mice exhibit lower colonization resistance compared to wildtype mice. Remarkably, I also observed rapid commensal microbe depletion as early as
D1 post- *C. rodentium* infection in the *Sigirr -/-* mice. My studies correlated their decreased colonization resistance with their enhanced secretion of antimicrobial peptides, and killing potential of the cecal crypts from the *Sigirr -/-* mice. The work described in this thesis is the first to link IEC innate responsiveness to the control of colonization resistance within the GI tract.

While colonization resistance is not a novel concept, the mechanisms that drive this effect remain unclear. Some researchers have suggested that the commensal microbiota modulate oxygen levels or short chain fatty acid (SCFA) levels within the intestine to suppress virulence gene expression by the invading enteric pathogen. This is based on Freter’s nutrient/niche theory that the number of ecological niches within the intestine is defined by nutrient availability [273,274,275]. Hence, if the resources or nutrients sought by the pathogen are already taken up by the commensal microbiota, the invading pathogens cannot colonize the GI tract. While Freter’s theory explains the basis of colonization resistance provided by the commensal microbiota, the molecular mechanisms have not yet been defined. Recently, a report by Winter et al. [226,276] suggested host derived factors such as nitrate benefit the growth of *S. Typhimurium* within the inflamed gut. These studies provide evidence that during intestinal inflammation, specific nutrients become available that are only usable by pathogens, and thus favour their growth. Notably, these studies are different from mine, in that they studied how overt gut inflammation can benefit pathogen growth. In contrast, my studies suggest factors prior to overt inflammation can benefit pathogen colonization and growth. While clearly the increase antimicrobial activity and rapid removal of competing commensals in the *Sigirr -/-* mice benefits pathogens, it is possible that the
intestines of *Sigirr*-/- mice contain nutrients such as tetrathionate that preferentially benefit pathogens. To address this issue, more work is required to determine whether there are any other factors or nutrients available in the intestines of *Sigirr*-/- mice that drive their increased susceptibility to infection.

### 4.2 Pathogen subversion of IEC inflammatory/antimicrobial responses

In many cases, for a bacterial pathogen to establish a successful infection within its host’s large intestine, it needs to disrupt the resident commensal microflora [14,24,25]. While direct interactions between pathogens and commensals likely occur, recent studies suggest that many pathogens accomplish this feat by triggering an inflammatory response by the host. In fact, this was first shown to occur during *C. rodentium* infection [64]. Moreover, aside from triggering a response that removes commensals, both *C. rodentium* and *S. Typhimurium* have demonstrated their ability to outgrow commensal microbes within an inflamed intestine [5,64]. However, there are potential drawbacks to this strategy. While most commensal microbes are poorly able to compete with pathogens within an inflamed environment, the strong inflammatory responses elicited by the host also damage the invading microbes [64,268]. Different enteric pathogens have therefore evolved distinct strategies to evade host inflammatory and antimicrobial responses. Intracellular pathogens such as *S. Typhimurium* protect themselves from the inflammatory response by residing within the host cells themselves. *S. Typhimurium* has developed bacterial effector proteins that subvert the maturation of phagosomes that would otherwise kill the invading microbes [277]. However, A/E pathogens, which do not invade host cells, have developed other strategies to survive host responses. Once EPEC and *C. rodentium* reach the intestinal mucosal surface, and they infect IEC, they subvert innate signalling by injecting T3SS-dependent effector proteins into
the IEC [26,27,88]. It should be noted that the suppression/hijacking of cellular responses only occurs in infected cells and not in neighbouring uninfected cells. As a result, EPEC and *C. rodentium* create a localized environment or niche that is immunosuppressed, and less hazardous to the pathogen than the nearby uninfected regions of epithelium.

### 4.2.1 A/E pathogen driven subversion of IEC responses

EPEC and *C. rodentium* deploy T3SS effectors that promote the ability of these pathogens to colonize their host and survive within the environment of the gut [26,27]. By injecting effector proteins into infected IEC, these enteric pathogens are able to suppress and hijack cellular functions to their own advantage. Furthermore, to ensure their success in modulating key host cellular responses, these pathogens often have more than one effector targeting the same signalling pathways [88]. Over the past few years, many groups, including ours, have shown that EPEC and *C. rodentium* suppress IEC inflammatory responses in a manner dependent on T3SS bacterial effectors [175,206]. During the time of this study, several research groups, including our own, identified NleC as one of the effectors involved in suppressing the IEC inflammatory response against EPEC infection *in vitro* [213,239,240,241]. The studies described in Chapter 3 furthered our understanding of the molecular function of NleC. I determined that NleC suppresses both p38 Map kinase and NF-κB in colonic epithelial cell lines. Both NF-κB and p38MAP kinase are important factors in controlling IEC production and release of the chemokine IL-8, as well as impacting on host cell apoptosis [278,279]. Furthermore, my study provided the first *in vivo* evidence that NleC suppresses inflammatory responses to A/E pathogens *in vivo*. We also noted that mice
infected with ΔnleC C. rodentium suffered more severe colitis than mice infected with wildtype C. rodentium.

Our development of a cecal loop infection model allowed us to test NleC as well as other effectors in a manner ensuring synchronization of the infections as well as equal pathogen burdens. Until now, most bacterial effector studies have been performed in vitro. While in vitro studies using IEC cell lines have great utility, they do not necessarily mimic the complexity of an intact host, with the dynamic interplay and signalling that occurs between different cell types. For example, once IEC are infected, aside from releasing antimicrobial peptides, they also release chemokines and cytokines to attract professional immune cells [176,280]. Our cecal loop model allowed us to examine the immunosuppressive effect of NleC in vivo as early as ten hours post infection. Although I did not observe overt immune/inflammatory cell infiltration into the infected tissues within this timeframe, I was able to detect a higher level of chemokine and cytokine gene expression in the ΔnleC C. rodentium infected tissues. The cecal loop model also enabled me to localize NleC expression within IEC following infection. The cellular localization of NleC within infected IEC had been in dispute, but through immunofluorescence staining, my work supported Yen et al’s finding that the NleC protein localizes beneath the pedestal at the site of bacterial attachment to the IEC [241]. Further study will be required however to determine exactly how NleC modulates cellular function by inhibiting both p38 Map kinase and NF-kb activation.
4.2.2 Multiple A/E pathogen effectors suppress IEC inflammatory responses

Over the last two years, several other EPEC T3SS dependent effectors have been identified as suppressing IL-8 release and modulating NF-κB activation within infected IEC. Aside from NleC, the effectors NleE, NleH1 and NleB were all shown to bind to NF-κB, hindering its activation and dampening IL-8 gene transcription *in vitro* [105,106,107,108]. It appears that A/E pathogens use these four effectors (and perhaps more) to inhibit inflammatory responses in IEC, by suppressing both NF-κB and p38 Map kinase signaling pathways. The observation that enteric pathogens such as EPEC and *C. rodentium* have acquired so many effectors to target the same pathways may be surprising, however as discussed in the introduction, NF-κB is a central molecule in regulating anti-microbial, inflammatory and apoptotic responses within IEC [169,279]. Similarly, p38 Map kinase is involved in stabilizing IL-8 gene transcripts and has been shown to play a critical role in recruiting T cells to the infected intestine during *C. rodentium* infection *in vivo* [262,278]. Therefore, it seems likely that EPEC and *C. rodentium* use multiple effectors to ensure these important signaling pathways are disrupted within infected IEC. Aside from inflammation, NF-κB and p38 MAP kinase dependent signalling also affects other host processes within IEC, such as cell death and repair. Elimination of infected IEC by shedding or apoptosis are likely critical factors in intestinal host defense [24,25]. If infected IEC are shed into the gut lumen, this also removes the pathogen from the host. Hence, the ability to suppress these two pathways would promote colonization of host intestines by EPEC and *C. rodentium*, by limiting both anti-microbial responses, as well as the removal of infected cells. Indeed, *ΔnleE* and *ΔnleH* strains of *C. rodentium* are impaired in colonizing mouse intestines when compared to wildtype *C.*
rodentium during competitive index studies [104,109], demonstrating that suppressing pro-inflammatory signalling within infected IEC is essential to the pathogenesis of A/E bacteria.

4.2.3 NleC as an anti-virulence factor

In contrast to the ΔnleE and ΔnleH C. rodentium mutant strains, which are outcompeted by wildtype C. rodentium, ΔnleC C. rodentium displayed a competitive advantage over wildtype C. rodentium. While we originally expected that impaired suppression of IEC inflammatory responses would reduce the competitiveness of the pathogen, as we have clearly laid out, this is not always the case. The basis for the competitive advantage carried by the ΔnleC C. rodentium strain is unclear, but host inflammatory responses have been previously shown to aid C. rodentium, as well as other enteric pathogens including S. typhimurium, in their colonization of the murine lower bowel [64,226]. The inflammation induced by ΔnleC C. rodentium might have aided in reducing colonization resistance at the mucosal surface. Since C. rodentium appears to initially colonize the intestine in a clonal fashion (ie. a single bacterium colonizes an IEC and then its progeny spreads from that site), localized differences in inflammation and pathologic changes in the gut mucosa could provide the mutant with a competitive advantage over the wildtype strain, promoting its spread through the host’s intestines. While the exact mechanisms underlying the competitive advantage of the NleC mutant are not immediately apparent, these findings do suggest that NleC might act as an anti-virulence factor. Anti-virulence factors have gained recognition recently, as have strategies used by pathogens to reduce/modulate their virulence to ensure the survival of their hosts, and their prolonged colonization of said hosts [281,282]. The exact basis for the anti-virulence effect of NleC is currently unknown, but it warrants further investigation.
4.3 Topics needing further assessment in my studies

Although the studies described in my thesis have begun to elucidate the mechanisms by which both host and pathogens can shift IEC inflammatory responses to their own advantage, there are several areas of research that require further assessment and analysis.

4.3.1 The cellular source of SIGIRR contributing to the observed phenotype

Based on the BM chimera studies outlined in Chapter 2, the cellular source of SIGIRR that limits IEC responses and promotes colonization appears to be non-hematopoietic/non-bone marrow derived cells. As SIGIRR is highly expressed by IEC, I examined whether it was SIGIRR expression within the IEC themselves that drove the observed phenotype. I isolated cecal crypts and examined the mRNA levels for several inflammation-associated genes. Taken together, the data suggest the cellular source of SIGIRR is the IEC. However, at present, my experiments have only shown correlative data and since my in vivo experiments were performed using mice lacking Sigirr in their germ line, it is possible that other cell types expressing SIGIRR may be contributing to the observed phenotype.

4.3.2 The definitive molecular mechanism remains elusive

While the studies performed in Chapter 2 have furthered our understanding of SIGIRR with respect to its role in maintaining mucosal integrity and homeostasis, the signalling pathways that are controlled by SIGIRR in our infection models remain to be fully defined. By crossing Sigirr -/- mice with other mouse strains lacking specific innate receptors, I confirmed that the Sigirr -/- mouse phenotype is dependent on MyD88, while the increased IEC proliferation and barrier function seen in these mice also depends on the IL-1R. However we still have to
address whether this reflects the actions of IL-1α, IL-1β or both. As well, through the removal of different innate receptors, I was not able to completely ameliorate the disease phenotype observed in the Sigirr -/- mice, therefore, there are other factors involved in leading to the exaggerated tissue damage. Furthermore, although we observed increased antimicrobial activity in the Sigirr -/- mice, it is not clear what causes the release of the antimicrobial factors. It does however appear that contact with a virulent pathogen may be required, since I did not observe an overt reduction of commensal microbiota when I exposed Sigirr -/- mice to an avirulent strain of C.rodentium (ΔescN). This suggests that the mere presence of a pathogen is insufficient to induce an exaggerated IEC antimicrobial/inflammatory response, but it must also possess and likely utilize its virulence factors, likely on the IEC that ultimately respond.

**4.3.3 Localization of NleC inside the cell**

One of the inconsistencies in those manuscripts published on the actions of the NleC effector is the cellular localization of NleC once it is injected into host cells [239,240,241]. I used a HA-tagged approach to visualize NleC and found that it localized below the pedestal both in vitro and in vivo. In contrast, other groups found that NleC was localized to the nucleus. While both results may be correct, it is also conceivable that the HA tag may have impaired or altered the normal trafficking of NleC, whereas the staining we performed was limited by detectable level of the HA-tag. Therefore it is possible that in our studies, some level of NleC might have been trafficked to the nucleus to directly interfere with NF-κB activation as previously reported.
4.4 Future directions

The work presented in this thesis answers some fundamental questions about the role of IEC in promoting host defense. However, this work also raises many new questions about the role of IEC in controlling pathogen-host interactions that we are actively pursuing. The following section of my thesis identifies these issues and how they may be addressed.

4.4.1 Determine the cellular source of SIGIRR contributing to the observed phenotype

As discussed in section 4.1.1 and 4.1.2, the work I performed in Chapter 2 provided strong evidence that loss of SIGIRR expression in IEC gives rise to the observed phenotype of the \textit{Sigirr} -/- mice, but other cell types could still be involved. To clarify whether loss of SIGIRR expression in IEC is the sole contributor to the observed phenotype, a tissue specific deletion of SIGIRR within IEC should be used. Ideally, \textit{Sigirr} floxed mice should be used to perform tissue specific deletion in IEC, as well as other cell types; however, at present, \textit{Sigirr} floxed mice are not available. We have started to generate \textit{Sigirr} -/- mice specifically lacking MyD88 within their IEC, to clarify the importance of innate signalling within IEC to the phenotype of the \textit{Sigirr} -/- mice. As seen in Chapter 2, the phenotype observed in infected \textit{Sigirr} -/- mice required MyD88 signalling. If SIGIRR expression in IEC is the sole contributor to the exaggerated tissue damage and reduced colonization resistance seen in \textit{Sigirr} -/- mice, then removing MyD88 signalling from the IEC of these mice should normalize their disease phenotype.
4.4.2 Explore the mechanisms involved in colonization resistance

As mentioned in section 4.1.3, the detailed molecular mechanisms that control colonization resistance remain elusive. Furthermore, aside from the increased production of anti-microbial peptides, it is uncertain whether there are other factors released by the host that result in reduced colonization resistance. In our laboratory we have started to examine whether abnormalities in pH, reactive oxygen species as well as nutrients or electrotransport systems may promote the colonization of the intestine by pathogens. As reported by Winter et al [226,276] host derived factors such as tetrathionate and nitrate can potentially benefit invading enteric bacterial pathogens. It was reported that S. Typhimurium can use tetrathionate, a by-product of releasing reactive oxygenated species, to support its growth under anaerobic respiration. Similarly, commensal E. coli was shown to utilize nitrate, generated as a by-product of the inflammatory response, for anaerobic respiration to gain a growth advantage. Thus, these by-products generated during intestinal inflammation benefitted invading pathogens to outcompete commensal microbes. It is uncertain whether these factors or other metabolites are present at high levels in the Sigirr -/- mice, either under baseline or infected conditions. Our laboratory has begun to explore this direction, and my colleague Dr. Martin Stahl; a post-doctoral fellow has begun to examine these factors using S. Typhimurium mutants that are impaired in the use of inflammation specific nutrients or cofactors, such as tetrathionate and nitrate.

4.4.3 Develop additional mouse infection models using Sigirr -/- mice

Evidence from Chapter 2 of my thesis suggests that Sigirr -/- mice can be colonized by enteric pathogens at doses (and under conditions) that are usually unable to colonize the
intestines of wildtype mice. This observation led me to test whether other enteric pathogens are able to infect Sigirr -/- mice. I infected Sigirr -/- mice with the human pathogen EPEC. As described in the introduction section, EPEC do not readily infect mice, hence the need to use *C. rodentium* to model EPEC infection [110]. Although the *C. rodentium* mouse model has provided valuable insights into mucosal immune responses against enteric pathogens, there are some key differences between EPEC and *C. rodentium* in their pathogenesis. For instance, EPEC express flagellin/flagella for motility, whereas *C. rodentium* are non-motile, and do not express flagellin [175]. Furthermore, although EPEC, EHEC and *C. rodentium* express similar T3SS-dependent effectors, the function of specific effectors appears to differ depending on their source. For example, Tir from EPEC versus EHEC has been shown to contain different structures and binding residues, thereby triggering different host signalling pathways during their respective infections [283,284]. Therefore, we felt it would be important and useful to develop a mouse model of EPEC to examine these effector functions *in vivo*. Remarkably, my preliminary data have shown that EPEC heavily colonizes the cecal and colonic epithelium of Sigirr -/- mice (Figure 4.2A), but not that of wildtype mice.

Furthermore, EPEC infection led to pedestal formation on the surface of intestinal epithelium of Sigirr -/- mice, as shown through transmission electron microscopy (Figure 4.2B). Based on my results so far, I am confident we can use EPEC infection of Sigirr -/- mice as a novel and relevant mouse model of EPEC infection. I have also started preliminary work examining the effectors involved in EPEC colonization in these mice. Interestingly, ΔnleC EPEC colonize the Sigirr -/- mice to a similar level as WT EPEC, but it induces more severe gut inflammation and pathology in the infected tissue. By infecting Sigirr -/- mice with various EPEC mutants, I expect we will develop a better understanding of the infection
dynamics of EPEC, which should help us develop better preventions and treatments against both EPEC and EHEC infections in humans.
Figure 4.2: *Sigirr* -/- mice are susceptible to EPEC infection.

*Sigirr* -/- mice were heavily colonized by EPEC D6 pi following streptomycin pretreatment. (B) Transmission electron microscopy revealed EPEC infected IEC of the *Sigirr* -/- mice and formed pedestal at the IEC surface. Transmission electron microscopy was performed by Dr. Wayne Vogl.
As demonstrated by my EPEC infection data, I believe Sigirr +/- mice can be used to establish new mouse models to study what are currently considered human specific enteric pathogens. For example, we could test the susceptibility of Sigirr +/- mice to the Gram-positive enteric pathogen Listeria monocytogenes and to other Gram-negative bacteria such as Campylobacter jejuni or adherent/invasive E. coli (AIEC). Our laboratory has already begun to explore this hypothesis, and my colleague Dr. Martin Stahl has generated preliminary evidence that Sigirr +/- mice are also susceptible to the Gram-negative pathogen Campylobacter jejuni.

4.4.4 Explore other potential functions of NleC

Although NleC has been described as a metalloprotease that can cleave the NF-κB p65 subunit, NleC has also been shown to bind the acetyltransferase p300 [285]. Moreover I have shown that NleC can dampen signalling from both p38 MAP kinase and NF-κB, suggesting NleC can modulate different cellular pathways, possibly by binding to different host cell factors. While the study by Baruch et al, explained that NleC affects NF-κB activity by cleaving the p65 subunit, it remains uncertain how NleC affects p38 Map kinase activation. Furthermore, it is possible that NleC affects other host cell functions, aside from inflammation. Considering the high homology between NleC and AIP56 - a bacterial protein encoded by Photobacterium damselae that drives apoptosis in macrophage and neutrophils [260,261,286], whether NleC affects host cell survival should also be examined. Finally, through competitive index studies, it appears that the ΔnleC C. rodentium possesses a competitive advantage over wildtype C. rodentium within the mouse gut, although the exact basis for this advantage is unclear. Identifying the potentially multiple binding partners for NleC will help us to elucidate other cellular functions regulated by NleC, and determine
whether the actions of NleC could potentially be used clinically to treat inflammatory diseases.

4.4.5 Examine the temporal requirement for pro-inflammatory and anti-inflammatory effectors expressed by EPEC and C. rodentium

Although Chapter 3 mainly outlined the immunosuppressive roles played by EPEC and C. rodentium T3SS-dependent effectors, other effector functions should not be overlooked. As discussed above, multiple effectors are involved in suppressing IEC inflammatory responses and other cellular functions. I feel it would be of interest to determine the hierarchy of effector expression or secretion through the T3SS of EPEC and C. rodentium, to further our understanding of infection dynamics. This could be achieved using a reporter system. It has been described Mills et al. [287] that in vitro, during EPEC infection, the amount and order of the LEE encoded effectors being secreted into infected cells is highly organized. However this study was performed in vitro and only focused on LEE encoded effectors. We have begun to address whether this analysis can be performed in vivo using live imaging techniques on infected animals. With the collaboration of Dr. Jose Puente from the National Autonomous University of Mexico, we have acquired tools to examine the induction of various T3SS dependent effector genes using luciferase reporter constructs. Our luciferase reporters are fused with the promoter regions of different LEE and Nle encoded effector genes to determine the expression profile of these genes. This type of approach will allow us to better understand the complex interplay between various effectors during infection. This approach has already been employed in collaboration with Dr. Gabriel Nunez showed that ler, a global regulator of LEE gene expression [288], is critical in allowing C. rodentium to outcompete commensal microbes [207]. It was shown that while Δler C. rodentium can
effectively infect germ free mice, the mutant cannot outcompete the microbiota to colonize normal mice. Currently there is no effective vaccine available to prevent EPEC infection, while treatment options remain rudimentary, ie rehydration therapy. My belief is that by developing a better understanding of the pathogenicity and infection course of EPEC, we will be able to design better therapies or vaccines against EPEC infection.

4.5 Final remarks

The findings reported in this thesis present evidence that both pathogens and the host proactively modulate inflammatory responses in the IEC to gain an advantage in the “arms race” between the bacterial and host interaction. Inflammation is a double edged sword that can lead to pathogen clearance, but it can also result in excessive tissue damage if not properly controlled. The role of IEC in providing host defense in the mammalian GI tract can now be expanded from acting as a primarily passive and hypo-responsive structural barrier to a more active cell type that promotes mutualism with the gut commensal microbiota to promote colonization resistance against pathogens. It has long been unclear why IEC are innately hypo-responsive to bacterial ligands \textit{in vivo}. My studies help to answer this question by revealing that negative regulators of innate signalling, such as SIGIRR limit IEC responses as a means to promote mutualism with commensal microbiota. Aside from the dietary benefits of such mutualism, the presence of commensal microbiota within the GI tract protects the host from pathogenic insults. On occasion, enteric pathogens are able to overcome colonization resistance provided by the commensal microbiota and infect the IEC. Once this occurs, to ensure successful and prolonged infection, pathogens such as EPEC and \textit{C. rodentium} suppress inflammatory responses by infected IEC using an array of effector proteins, including NleC. It is hoped that the studies outlined in my thesis will generate new
interest and insights into the function of the IEC layer, and its importance in promoting intestinal health.
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