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Abstract

Diarrheal disease caused by attaching and effacing pathogens, such as enteropathogenic *E. coli* (EPEC), is a worldwide health concern. As the second leading cause of diarrheal-related death in young children, new investigations into host defense against EPEC, as well as future therapeutics, is greatly needed. To elucidate the host immune responses to these enteric pathogens, the attaching and effacing (A/E) murine pathogen, *Citrobacter rodentium,* has been widely used. It is well understood that *C. rodentium* infection induces a robust Th1 response within the host. Yet how these pleiotropic IFNγ immune responses are initiated, propagated, and the accessory immune cell types involved remains poorly understood. In this thesis, I investigated how innate immune cell types such as natural killer cells, which are significant producers of IFNγ, mediate these Th1 directed responses. This work identified that both NK and NK-like innate lymphoid type 1 cells (ILC1s) are capable of producing IFNγ in response to *C. rodentium*, and NK cells rapidly increase in numbers within the colon during the early stages of infection. Depletion of these cell types causes a delayed Th1 CD4+ T cell response within the colon, resulting in increased bacterial load, and greater degree of colonic pathology at later time points. Additionally, depletion of these cells results in decreased CXCL9 chemokine expression in mice. I later determined that CXCL9 exhibited direct antimicrobial action against *Citrobacter in vitro*. Depletion of this chemokine *in vivo*, in the absence of adaptive immune responses, or its receptor CXCR3, results in increased mortality rates, elevated bacterial loads, greater degree of pathology, and deeper penetration of bacteria within the colonic crypts. These data indicate a

potential direct antimicrobial role for this IFNγ-induced chemokine, independent of its known properties for the homing of T cells to the site of infection. These findings demonstrate the importance of accessory IFNγ-producing immune cells in not only mediating Th1 CD4+ T cells responses, but also other innate host defense mechanisms against A/E pathogens.

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List of Abbreviations

A/E	attaching and effacing
ADCC	antibody-dependent cellular cytotoxicity
aEPEC	atypical enteropathogenic *E. coli*
AIEC	attaching and invasive *E. coli*
AMP	antimicrobial peptide
ANS	8-anilino-1-naphthylenesulfonic acid
APC	allophycocyanin
APC	antigen presenting cell
ATEC	atypical enteropathogenic *E. coli*
B6	C57BL/6
BGA	Brilliant Green agar
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CD	cluster of differentiation
CFU	colony forming units
CHS	contact hypersensitivity
CI	cellular infiltrates
C. rodentium	*Citrobacter rodentium*
Cri1	*Citrobacter rodentium infection locus 1*
D	desquamation
DAEC	diffusely adhering *E. coli*
DC	dendritic cell
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
E	edema
EAEC	enteroaggregative *E. coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
ELISA	enzyme-linked immunosorbent assay
ELR	glutamate-leucine-arginine
Eomes	eomesdermin
EPEC	enteropathogenic *E. coli*
E. coli	*Escherichia coli*
Esp	*E. coli* secreted protein
ETEC	enterotoxigenic *E. coli*
FACS	fluorescent-activated cell sorting
FAS	fluorescent-actin staining test
FBS	fetal bovine serum
FiTC	fluorescein
GAS	Gamma associated sequence
GATA	Trans-acting T-cell-specific transcription factor

GATA3	GATA binding protein 3
GC	goblet cells
H	colonic hyperplasia
H_2SO_4	sulfuric acid
H&E	hematoxylin and eosin
HD5	human α-defensin 5
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hk	heat killed
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
ID2	inhibitor of DNA binding 2
IEC	intestinal epithelial cell
IFN	interferon
IFNγR	interferon gamma receptor
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
IMEM	Iscove's modified eagle media
iNK	immature natural killer
IP-10	interferon gamma-induced protein 10
IRF	interferon regulatory factor
ITAC	interferon-inducible T-cell alpha chemoattractant
Jak	Janus kinase
KIR	killer Ig-like receptors
KLR	killer cell lectin-like receptor
L	lumen
L-glut	L-glutamine
LB	Luria-Bertani medium
LEE	locus of enterocyte effacement
LP	lamina propria
LTi	lymphoid tissue inducer
M	mucosa
M1	classically activated macrophage
M2	alternatively activated macrophage
MCMV	murine cytomegalovirus
MCP	monocyte chemotactic protein
mCRAMP	murine cathelidicin-related antimicrobial peptide
MHC	major histocompatibility complex
MIG	monokine induced by gamma interferon
MIP	macrophage inflammatory protein
MLN	mesenteric lymph nodes
mNK	mature natural killer
mRNA	messenger ribonucleic acid
NaCl	sodium chloride

NCR	natural cytotoxicity receptors
NK	natural killer
NKG2D	natural killer group 2 member D
NKP	natural killer cell progenitor
Nle	Non-LEE encoded
NLR	Nod-like receptors
NMEC	Neonatal meningitis/sepsis causing *E. coli*
Nod	nucleic acid oligomerization domains
OCT	optimal cutting temperature compound
OD	optical density
PBS	phosphate-buffered saline
PE	R-phycoerythrin
Penn/Strep	penicillin/streptomycin
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
PRR	pattern recognition receptor
RAG	recombination activating gene
RANTES	regulated on activation, normal T cell expressed and secreted
Reg	Regenerating islet-derived protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SE	surface epithelium
SEM	standard error of the mean
SIGIRR	Single Ig IL-1 related receptor
SM	submucosa
SOCS	suppressor of cytokine signaling
Stat	Signal transducer and activator of transcription
T3SS	type III secretion system
Tbet	T-box expressed in T cells
Th	T helper
TLR	Toll-like receptor
TMCH	transmissible murine colonic hyperplasia
TNF	tumor necrosis factor
Treg	regulatory T cell
UPEC	Uropathogenic *E. coli*
WT	wild type
γ_c	common γ-chain receptor

Chapter One

Introduction

Chapter One – Introduction

Infectious Diarrheal Disease, An Overview

Infectious disease has troubled human kind since the dawn of our existence. The advent of antibiotics, as well as vaccination, led Sir Frank McFarlane Burnet, in 1951, to famously state that, "most all practical problems of dealing with infectious disease were solved" [1]. Many were hopeful that he would be correct in his prediction. Yet, in the 21st century, humanity is facing rampant antibiotic resistance, and the continual death of millions of people every year due to infectious disease. In particular, gastrointestinal infections are one of the leading causes of death in early childhood. In the early 1980s, global estimates determined that diarrheal illnesses attributed to the death of more than 4-6 million children every year, with the highest mortality rates in developing nations. Those numbers have fallen within the past several decades to an estimated 2-5 million deaths per year—a decline mostly due to the implementation of oral rehydration therapies by the World Health Organization [2]. However, continued research into furthering our understanding of how enteric bacterial pathogens infect their hosts, cause disease, as well as potential new treatment protocols is greatly needed.

Pathogenic E. coli

Escherichia coli is a gram-negative bacterium that is major colonizer of the human gastrointestinal tract [3-5]. The bacterium has been found to colonize humans within hours of birth, and from its discovery in 1885 by Dr. Theobald Escherich until the mid-1900s, *E. coli* was largely considered to be a strictly commensal bacteria of human feces [6,7]. Then in the 1940s, reports from Bray, and Bray and Beaven, implicated *E. coli* as the causative agent in several cases of infantile diarrhea [8,9]. Since these discoveries, multiple new pathovars of *E. coli* have been identified as the causal agents of infectious diarrhea, where bacteria are either restricted to the primary mucosal site, or capable of systemic infection. Other pathogenic strains of *E. coli* have also been indicated to cause urinary tract infections and, more recently, sepsis and meningitis. To date, ten distinct pathogenic types of *E. coli* have been identified: uropathogenic *E. coli* (UPEC), neonatal meningitis/sepsis causing (NMEC), adherent invasive (AIEC), enteroaggregative (EAEC), diffusely adhering (DAEC), enteropathogenic (EPEC), atypical enteropathogenic (aEPEC or ATEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), and enterotoxigenic (ETEC) [10-12]. These pathovars have been categorized based on distinct virulence factors, and characteristics and pathology of disease within the host. Clonal groups are determined within these pathogens through their serotype O (somatic), and H (flagellar) antigens [12]. However, several strains have recently been identified whose typing bridges two or more pathovars, indicating that pathogenic *E. coli* bacteria continue to evolve [13].

Enteropathogenic E. coli

Of all of the pathogenic *E. coli* pathovars, enteropathogenic *E. coli* (EPEC) infections are the leading cause of diarrheal-related death in children under 5 years old and are secondary only to rotavirus infections within this category [14,15]. With a mortality rate as high as 40%, a significant presence within both community and hospital settings, and recent studies revealing that children infected with EPEC are more likely to fail rehydration treatments, a deeper understanding of host-pathogen interaction and development of new treatments is critical for combatting the continued loss of young life [2,14].

After its discovery as the causal agent of infantile diarrheal disease in the 1940s, controversy emerged regarding its classification as a pathogen due to a general inability to reproduce similar pathologies experimentally. It was not until the 1970s, when human subjects were voluntarily dosed with high CFU inoculums of EPEC, were researchers able to recapitulate the phenotypes originally attributed to this strain of bacteria [16,17]. A typical EPEC infection results in diarrheal symptoms, which have a high likelihood of transitioning from acute to persistent diarrhea [2,14]. Infection can be accompanied by fever, rash, and vomiting, although profuse watery diarrhea, and severe gastroenteritis are the true clinical markers of the disease [14]. As a pathogen, EPEC has adapted itself to colonizing the proximal small intestine of larger mammals, adhering to the apical plasma membrane of epithelial cells at this site. After initial adherence, EPEC form intimate attachments with the epithelial cell mediating pedestal-like formations, and loss of

microvilli [18]. These pedestal formations have become the true hallmark of an EPEC-driven infection, and led to the development of the fluorescent-actin staining test (FAS), the standard clinical test for confirming EPEC infection [19].

Attempting to understand the mechanism behind pedestal formation by EPEC and other attaching and effacing pathogens, and why this is such a critical feature required for virulence, has been a primary research focus of many groups investigating this pathogen. While the precise benefit to the pathogen in the formation of these pedestals still remains elusive, a greater understanding of how the bacterium is able to hijack host cellular processes in this manner has gained some clarity. However, an inability to reproduce several of these *in vitro* findings within larger animal models, or human colon explants has revealed a need for an *in vivo* model system for the study of EPEC, and other A/E pathogens [20-22]. While EHEC has shown some promising data using weanling lambs, or rabbits, there still does not exist a robust small animal model for EPEC, or EHEC infections [22]. Therefore, many researchers utilize the strictly murine pathogen, *Citrobacter rodentium*, another A/E pathogen family member, to delve into both mechanisms of disease and host responses to EPEC, as well as EHEC. *Citrobacter* has shown great promise in this way, and has been found to recapitulate many of the *in vitro* findings previously reported for the human *E. coli* pathogens [12,23].

Citrobacter rodentium model

Citrobacter rodentium is a natural mouse pathogen of the *Enterobacteriaceae* family [24,25]. *C. rodentium* was initially discovered in the 1960s as the infectious agent responsible for an outbreak within an animal facility. Originally mistaken for a strain of *Citrobacter freundii* biotype 4280, it was later found to be a unique organism of the *Citrobacter* genus, and was termed transmissible murine colonic hyperplasia (TMCH) due its pathology of hyperplasia of colonic crypts, prior to being renamed *Citrobacter rodentium* [26]. *C. rodentium* is highly transmissible via the fecal-oral route: only a single infected mouse is necessary to infect an entire cage of uninfected mice [27]. Like EHEC and EPEC, *C. rodentium* possesses the 35 kb locus of enterocyte effacement (LEE) pathogenicity island [28,29]. The LEE pathogenicity island is required for formation of a type III secretion system (T3SS), which is critical for bacterial attachment to epithelial cells, and the formation of identical pedestal-like structures as EPEC, and EHEC. Formation of pedestals, as well as the secreted effectors of the T3SS, is necessary for the bacteria to commence, and sustain infectivity within the host [29]. Infection is generally limited to surface mucosa of the colon, and at lower levels in the cecum, with bacteria rarely reaching systemic sites [26]. The typical course of infection upon oral inoculation commences with a primary infection of the cecum, where it acclimatizes itself to the host prior to spreading to the colon [30]. This acclimatization delay is dependent upon luminal bacteria, as germ-free mice do not experience any delay in colonization of the intestinal tract [31-33]. Once acclimatized, *Citrobacter* efficiently outcompetes the host microbiota

within the colon, replacing up to 90% of the commensals at this site [34,35]. Bacterial numbers continue to mount until burden within the colon reaches its peak approximately one week after primary inoculation, with peak of inflammation following 3-7 days later. Initiation of clearance at two weeks is observed in resistant mice, with complete clearance of *C. rodentium* by 3 to 4 weeks. Intestinal pathology from the infection, however, can remain visible for up to 8 weeks [26].

Severity of infection with *Citrobacter* is dependent upon multiple factors, such as age, host genetics, supplier sources, diet, and microbiota composition. It has been well established that weanling mice, as well as neonates, exhibit increased susceptibility and generally succumb to *Citrobacter* infection. It is weanling and neonates that represent the greatest loss during outbreaks in animal facilities. However, some strains of mice, independent of age, also succumb to infection [32]. Resistant strain, C57BL/6, exhibit what is considered typical infection dynamics, as described above. Susceptible strains C3H and FVB mice have a 50-100% increase in mortality, and typically succumb to infection within the first two weeks of infection. Mortality in C3H and FVB strains is associated with increased bacterial load, weight loss, as well as pronounced inflammation resulting in necrosis and erosion of the intestinal barrier. Dehydration has been found to be the main cause of mortality within these strains, as fluid therapy eliminated this phenomenon [36,37]. Decreased expression of several genes involved in ion transport across the intestinal epithelium was observed in susceptible strains [36,37]. However, cross-breeding of resistant and susceptible mouse strains, as well as congenic mouse studies revealed that a unique locus called *Citrobacter rodentium infection 1* (*Cri1*), and

not any direct genomic variation within transport genes, was critical for survival in resistant mice. Replacement of a susceptible FVB *Cri1* allele with resistant C57BL/6 *Cri1* allele successfully eliminated mortality in FVB mice. This protective nature of the C57BL/6 *Cri1* locus was found to be genetically recessive as even a single copy of C3H or FVB susceptible allele led to increased mortality [38,39]. Recent work by Papapietro *et al*. revealed that R-spondin 2, encoded within the *Cri1* locus, was overexpressed in the colons of susceptible mice leading to an unchecked proliferative response within colonic crypts [40]. This increased rate of proliferation led to poorly differentiated crypt epithelial cells with deficiencies in ion transport components, which are essential for controlling diarrheal severity, and maintaining host hydration during infection [36,37,40].

While survival is dependent upon age and mouse strain, mouse supplier, diet, and microbiota composition additionally influence disease pathology. Many groups have reported differential disease severity in mice of identical genetic background, but different supplier sources. Many hypothesize that this is due to the inbreeding of not as yet identified genetic abnormalities, diet, and altered microbiota compositions. Differing diets can result in variable colonic crypt heights in both infected and uninfected mice, with some infected mice displaying increased hyperplasia [32]. Recently it has been shown that mice fed diets deficient in either vitamin A or D have increased susceptibility to *Citrobacter* infections [41,42]. Diet-directed increased susceptibility, whether due to vitamin deficiency, or other dietary interventions, has been linked to altered intestinal epithelial cells (IECs) signaling, deficient antimicrobial responses, epithelial regeneration, and skewed immune cell responses [41-47].

Diet has been shown to not only alter IEC and immune cell responses, but to also influence microbiota composition of mice; and these alterations can affect susceptibility to *Citrobacter rodentium* [47,48]. The host microbiota plays a critical role in controlling the rate, and ability of *Citrobacter* to colonize. Exchange of luminal contents through fecal transplants from resistant to susceptible strains of mice has been shown to be protective [49]. As well, prophylactic delivery of bacteria of the genus *Lactobacillus,* or *Bifidobacterium,* prior to infection can offer protection against *Citrobacter*, limiting its ability to colonize [50-54]. The bacterial milieu of the intestinal tract not only affects a pathogen's capacity to colonize the intestinal lumen, but also strongly influences clearance of *Citrobacter* from the colon through competition by commensals who share similar metabolic niches [33].

Type 3 Secretion System

While age, strain, and microbiota composition of the host can all affect colonization, clearance, and survival, the ability of the bacteria to be pathogenic in mice relies on an arsenal of bacterial virulence factors. The main virulence strategy used by *C. rodentium*, as well as human A/E pathogens, is the type III secretion system (T3SS) encoded within the LEE pathogenicity island [28]. Type III secretion systems are critical for translocation of bacterial proteins into host cells and present a common virulence strategy employed by various Gram-negative pathogens [55,56]. Loss of a functional T3SS, whether by deletion of the entire LEE island or through deletion of key

components, renders *C. rodentium* avirulent [29]. These complex protein export machines are multi-component systems with a needle-like component that spans the bacterial outer membrane, extracellular space, and the host membrane [57]. While a large number of bacterial proteins are secreted by T3SS, there is an evident hierarchy that determines the order in which specific protein substrates are secreted. The primary substrates are the needle and inner rod components. Once constructed, the needle secretes the translocators, which are required for translocation of bacterial protein effectors into the host cells. Finally, the effectors themselves are secreted into the host cell [58].

Effectors

Effectors are proteins secreted by bacteria that enable them to subvert or modulate host immune and cellular responses; therefore, forming vital components in the bacteria's ability to colonize a host, as well as manipulate disease outcomes. In *Citrobacter rodentium*, as well as other A/E pathogens, effectors are designated as either encoded within the LEE island as *E. coli* secreted proteins (Esp), or non-LEE encoded effectors (Nle); however, these designations are not universal throughout the literature with either Nle effectors having Esp designations, or effector nomenclature which is more indicative of their cellular functions. Genomic mining studies in A/E pathogen genomes revealed over 200 potential effectors in EHEC, and over 400 in EPEC, however, only 39, and 21, respectively, were found to be secreted [59]. The number of secreted effectors appears to be strain specific, as different EPEC strains can range in secreted effector numbers from

21 to 40 depending on the strain examined. *C. rodentium* has 30 secreted effectors [60]. While A/E pathogens share a common T3SS and many core effectors, there does appear to be plasticity within their effector repertoire encoded outside of the LEE island, and likely are the reasons for the variation of diseases caused by these bacteria. However, they do share 21 core effectors, which are critical for generating the hallmark features of A/E pathogen-induced infection [60].

Determination of effector-specific functions within the host is not a straight forward process as many effectors not only have pleiotropic effects within host cells, but also can have redundant roles shared with multiple effectors. In addition, several effectors have been shown to antagonize other effectors, dampening or altering their effects [23]. However, several decades of research, both *in vitro* studies with EPEC, and *in vitro* and *in vivo* studies with *C. rodentium*, have revealed specific phenotypes, and cellular targets for a number of the 21 core effectors. This is a continually evolving field, as new effectors continue to be uncovered, as well as new potential interaction partners for known effectors. To date, the main host cellular responses manipulated by *Citrobacter* and other A/E pathogens are actin dynamics, diarrheal responses, apoptotic pathways, and inflammatory responses [23].

Innate Host Defenses

For gastrointestinal pathogens, in particular A/E pathogens, where bacteria enter hosts via oral ingestion, primary host innate defenses begin with the gastric juices within

the host stomach [61]. Pathogens that are capable of withstanding the low pH of the stomach, must then outcompete commensal microbes within the gut, penetrate the mucus layer, and then attach to and/or invade epithelial cells. The mucus layer provides an essential protective barrier, deterring both potential invading pathogens, as well as commensal bacteria. It is partitioned into two distinct environments; with commensal bacteria heavily colonizing the outer region, whereas the firmly attached inner region, adjacent to the epithelial barrier, is largely sterile, and lacking evidence of bacterial colonization [62]. Epithelial mucins, with Muc2 as the most prevalent form, are produced by goblet cells throughout the small and large intestines [63-66]. Loss of Muc2 production, and therefore a functionally protective mucus layer in muc2-/- mice, increased susceptibility to colonization of the colonic epithelium not only by *Citrobacter* but also commensal bacteria, and these mice quickly succumbed to infection [67,68]. However, the mucus layer may not be solely protective structure as preliminary evidence suggests that *C. rodentium* may utilize the mucus layer as a nutrient source. For instance, in mice lacking Muc2, *Citrobacter* is no longer able to outcompete commensals; as well, *C. rodentium* preferentially associates with crypts where goblet cells have not been depleted [68,69]. Indeed, prevention of goblet cell depletion through chemical intervention results in increased bacterial burden, and mortality in treated mice [70]. Therefore, the mucus layer, and in particular Muc2 production, provides initial protection from invading pathogens, as well as commensal bacteria; conversely, it also may be a significant nutrient source for invading pathogens.

Once bacteria have penetrated both regions of the mucus layer and attached to the apical epithelial surface of the intestinal tract, the primary responses of IECs are to induce diarrhea, proliferation, and sloughing of IECs, as well as the secretion of antimicrobial and pro-inflammatory factors; therefore, attempting to rid the intestinal tract of infected cells, as well as extracellular pathogenic bacteria. The ability of IECs and immune cells to recognize invading pathogens and induce an appropriate immune response is dependent upon pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), Nod, and Nod-like receptors (NLRs), expressed within the outer membrane, intracellular vesicles, or within the cytosol of the host cells [71]. These receptors recognize and bind to various particles common to viruses, bacteria, parasites, etc. The recognition of which triggers various inflammatory signaling cascades and the expression of antimicrobial factors, as well as cytokines and chemokines that aid in directing the inflammatory milieu of the *Citrobacter*-infected colon, which is typically defined by Th1 (T helper type 1), and Th17 responses [72,73]. The specifics of the Th1 CD4+ T cell response during *C. rodentium* infection and the accessory immune cells necessary for this response will be discussed in detail within later sections.

Epithelial cells cannot, however, sustain inflammatory responses for long without risking complete degradation of the epithelial barrier, so IECs must also initiate tolerogenic responses in order to survive prolonged exposure to *C. rodentium*. While losses of various inflammatory responses have been found to be critical for host survival and bacterial clearance, tolerogenic responses have also been shown to play a critical role not only in maintenance of the mucosal barrier, but also in host survival. Tolerogenic

TLR2-directed signaling is known to be essential for protection of the mucosal barrier, particularly with dampening the impact on epithelial cells of immune cell TLR4–directed inflammatory responses [74,75]. In addition, Single Ig IL-1 Related Receptor (SIGIRR), a negative regulator of IL-1 and TLR signaling, has also been shown to be a critical for dampening inflammatory responses within the epithelium, and is protective in limiting at least the initial inflammatory responses which *Citrobacter* requires to outcompete the luminal commensal populations [76]. Secreted factors, such as the cytokine IL-6 (Interleukin 6), and various antimicrobials such as RegIIIγ, that have also been shown to be critical for mucosal protection, and host survival [77,78].

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are an essential part of epithelial, and immune cell defense strategies against invading microbes, as well as maintenance of commensal populations at mucosal surfaces within the host. Diverse both in structure and mechanism of action, over 2000 AMPs have been isolated from a variety of sources ranging from bacteria and plants, to insects and humans [79]. In general, AMPs can be segregated into four categories based upon their predominant secondary structure: α-helical, β-sheet, mixed α-helix/β-sheet, and linear/extended [80]. AMPs can also be segregated into anionic, and cationic antimicrobial peptides, with the vast majority of known AMPs being cationic. Cationic antimicrobial peptides have been the primary focus of research into the potential use of AMPs as future therapeutics, due to their innate ability to associate with

negatively charged bacterial membranes, while avoiding the neutrally charged cell surface of host cells [81,82]. The mechanism of action of cationic AMPs has been the source of some debate, for while it is known that these peptides associated with bacterial membranes, it is unclear as to how, and if this interaction mediated their bactericidal effects. There are presently three models for AMP interaction with bacterial outer membrane and each offer alternative mechanisms by which AMPs form pore-like structures within the bacterial membrane: barrel-stave, carpet, or toroidal pore models [83]. Current thought suggests that a multistep process occurs where peptides bind to the bacterial outer membrane surface, re-align and then penetrate further into the membrane, eventually resulting in membrane rupture, or leakage of cellular contents through AMP-formed pores [84]. Alternatively, there is also evidence that some AMPs can mediate their antimicrobial activity through interaction with membrane-associated proteins, or targets within the bacterial cytoplasm [85]. In addition to bactericidal function, some AMPs have also been shown to be immunomodulatory [86,87]. For instance, human α-defensin and β-defensin members exhibit chemotactic properties, which mediate some of the previously observed antibacterial activities of these AMPs [88]. Immunomodulatory effects of AMPs are not, however, limited to chemotactic properties, as many have been shown to participate in wound healing, inflammation, and adaptive immune responses [86].

AMPs and Citrobacter

Antimicrobial peptides can be expressed by a wide variety of cells within the body, both constitutively, and inducible. Induction of AMPs requires activation of pattern recognition receptors, as well as cytokine stimulation of the cellular source. Multiple cytokines have been shown to be critical for protective AMP expression during *C. rodentium* infection. In particular, IL22 produced by ILC3s (innate lymphoid cells type 3) has been shown to mediate epithelial expression of several AMPs (RegIIIβ, RegIIIγ, S100A8, S100A9, and lipocalin 2) in response to *C. rodentium* infection [78,89]. Mice lacking IL22 quickly succumb to infection, and delivery of exogenous RegIIIγ is able to prevent mortality in these mice [78]. In addition to IL22-stimulated AMPs, IFNγ has been linked to increased levels of murine CRAMP (LL-37 in humans), and β-defensins [90-92]. While the loss of CRAMP in mice does not impact survival, bacteria are able to penetrate further into crypts compared to wild type mice where bacteria tend to persist along the outer apical surface of the epithelium [93]. It is unknown whether β-defensins function in a similar capacity. In addition to these AMPs, IFNγ also induces the expression of several chemokines, CXCL9, CXCL10, and CXCL11, known to possess antimicrobial effects independent of their chemotactic abilities [94].

Chemokines as AMPs

Chemokines are a superfamily of small proteins (8-14kDa) that are critical for immune cell homing in a concentration-dependent manner [95,96]. Classification of these proteins into four distinct subgroups is dependent upon arrangement of the conserved N-terminal cysteine motifs. These subgroups are C, CC, CXC, and CX_3C, where the X represents an amino acid other than cysteine [97]. Chemotactic capabilities of chemokines are dependent upon their respective receptors, but this interaction is relatively promiscuous as receptors can bind several chemokine ligands, just as chemokines may bind multiple receptors [98]. Additionally, there is redundancy in immune cell responses directed by chemokine-receptor interactions, as no one chemokine is essential for the homing of a single leukocyte in every scenario.

In addition to their chemotactic properties, many chemokines have been shown to exhibit direct antimicrobial properties *in vitro* [99-101]. Currently, 23 (13 CC and 10 CXC) out of 45 human chemokines have been shown to be antimicrobial against a variety of Gram-negative and Gram-positive bacteria [102]. Chemokines that exhibit antimicrobial activity tend to share similar amphipathic structural features as cationic AMPs; characterized by an accumulation of positively charged residues, as well as distinct separation of hydrophobic, and hydrophilic residues [103,104]. Investigation into the potential *in vivo* antimicrobial effects of these chemokines has been hindered by the difficulty in separating chemotactic and antimicrobial effects. Recently, however, chemokines, CXCL9, CXCL10, and CXCL11, were shown *in vivo* to have direct

protective effects against *Bacillus anthracis*, independent of their receptor, CXCR3, revealing potential antimicrobial action for these chemokines *in vivo* [105].

Interferon-γ

Interferons (IFN) were first discovered in 1957, when influenza-infected chick chorioallantoic membranes were found to produce a soluble factor that 'interfered' with viral replication [106]; thus, leading to the classification of these proteins as interferons. Therefore, when IFNγ was initially discovered to inhibit viral replication it too was classified as an interferon, although later structural analysis of the protein revealed it shares greater similarity with interleukins [107,108]. Despite these findings, IFNγ continues to be classified as an interferon, though it has been relegated to its own family of interferons known as Type II. IFNγ is secreted by a number of innate and adaptive immune cells, with natural killer (NK) cells, γδT cells, NKT, CD8+ T cells, and Th1 CD4+ T cells being the largest producers of the cytokine [109-113]. The ability to secrete IFNγ is not, however, limited to these cell types as mounting evidence has revealed that, given the appropriate stimuli, monocytes/macrophages, dendritic cells (DCs), B cells, and recently, even neutrophils are capable of expressing this cytokine [114-116]. Beyond its role in viral defense, IFNγ has been shown to play important roles in bacterial defense, antigen processing, immunoglobulin (Ig) class switching, adhesion, leukocyte homing, cell cycle regulation, apoptosis, tumor immunity, and autoimmunity [117-119]. Many of these functions are critical for host defense, as humans lacking IFNγ, its receptors, or key

signaling components, display increased susceptibility to both viral and bacterial infections [120-122].

In order to perform its numerous functions, IFNγ, which functions as a homodimer, is secreted and signals in both an autocrine and paracrine manner through its receptor, IFNγR. The IFNγR consists of two homodimer subunits, IFNγR1 and IFNγR2 [123]. IFNγR1 is constitutively expressed, while IFNγR2 is available only in limited amounts, and its induction appears to control a cell's responsiveness to IFNγ [124,125]. Upon binding to its receptor, IFNγ predominately signals through the Jak/Stat pathway, in particular Jak1/2 (Janus kinase 1 and 2) and Stat1 (Signal transducer and activator of transcription) [119]. Activated Stat1 translocates to the nucleus, where it initiates transcription for genes possessing a defined DNA sequence known as GAS (Gamma associated sequence) within their promoter region [117]. Stat1-independent, or non-classical activation of GAS genes has also been observed, as one third of these genes are still capable of being expressed in a STAT1 deficient mouse and appear to rely upon various alternative signaling pathways. Interferon regulatory factors (IRF) family members (IRF1, 2, and 9), and SOCS (suppressor of cytokine signaling) proteins are also involved in positively, and negatively regulating IFNγ signaling [126-128].

Classically considered a proinflammatory cytokine, due to its ability to activate macrophages, and direct Th1 cell-mediated immunity both of which will be discussed in later chapters, IFNγ can also mediate regulatory, as well as anti-inflammatory effects. These effects are achieved through initiation of apoptotic pathways in activated T cells, limiting Th17 responses, as well as aiding in the differentiation of regulatory T cells [129-

134]. The latter was found to be of particular importance in collagen-induced arthritis, where IFNγ deficient mice exhibited limited populations of antigen-induced CD4+ regulatory T cells (Tregs). The timing of these protective effects tend to be in the later stages of disease progression, as neutralization of IFNγ responses early on can be protective against inflammation-induced pathology, whereas similar interventions later appear to exacerbate tissue damage [129,135].

Previously, IFNγ was known for its essential role in host defense against viral, and intracellular pathogens. Recently, however, a protective role has also been observed in extracellular pathogens, particularly in the necessity for IFNγ in the generation and protective responses of Th1 CD4+ T cells. Reliance upon host IFNγ responses in susceptibility to extracellular pathogen, *C. rodentium*, has been observed in IFNγ deficient mice, as these mice exhibit delayed bacterial clearance, exacerbated infection-associated pathology, and diminished antibody responses [90,136].

Natural Killer Cells

Natural killer (NK) cells are large granular lymphocytes that form an essential branch of the innate immune system. Originally discovered in 1975 for their unique ability to directly kill tumor cells without prior sensitization, NK cells were long thought to be the only innate cell type of lymphoid origin [137]. However, in the past several years, many have come to acknowledge that NK cells are the founding members of a family of innate lymphocytes now termed innate lymphoid cells (ILCs). This family of innate

lymphocytes shares several common features: a typical lymphoid morphology, absence of both myeloid or dendritic cell (DC) markers, as well as a lack of recombination activating gene (RAG)-dependent antigen receptors [138,139]. A distinct common progenitor of ILCs has not yet been identified; however, it has been observed that ILCs depend upon the common γ-chain receptor (γ_c), and transcriptional repressor inhibitor of DNA binding 2 (ID2) for their development [138,140]. ILC nomenclature has evolved to include three distinct groups, designated 1 through 3, which are segregated based upon cytokine secretion, and expression of specific transcription factors. Group 1 ILCs include NK cells and ILC1s, and are defined by their ability to secrete high levels of IFNγ, and expression of Th1 cell-associated transcription factor T-bet. Group 2 ILCs produce Th2 cell-associated cytokines such as IL-5, and IL13 and are dependent upon the transcription factors GATA-binding protein 3 (GATA3), and retinoic acid receptor-related orphan receptor α (RORα). Group 3 ILCs are defined by their ability to produce IL-17, and/or IL-22, and share not only similar cytokine production as Th17 cells, but also Th17 transcription factor RORγt [141,142]. The latter group is of particular significance in *C. rodentium* host defenses, and their protective effects are mediated through IL-22-induced IEC expression of antimicrobial, RegIIIγ [78,143,144].

ILCs have wide range of innate functions such as defense against pathogens, epithelial barrier function, tissue repair, and generation of secondary lymphoid structures, such as lymph nodes, and immune nodules within the intestine [139,145]. Natural killer cells, while representing only a singular branch of the innate lymphoid cells, have come to be known as the “jack of all trades” immune cell. Their vast repertoire of functions

include defense against both pathogens and aberrant host cells, as well as regulatory roles in placenta formation, homeostasis of immune responses, and shaping of adaptive immune responses [146-148].

Prior to birth, NK cells develop within the fetal liver. They are quickly lost from this site shortly after birth, and the bone marrow takes over as the predominate source of NK progenitors [149]. With the discovery of various subsets of ILCs within recent years, a renewed interest in the development of NK cells, as well as ILCs in general, has shed new light on the various stages of their development from common lymphoid progenitor to mature conventional NK cell [138,140,150]. Initial differentiation from the common lymphoid progenitor to pre pro NK progenitor (NKP) cells is the acquisition of CD127. The transition from NKP to immature NK (iNK) cell involves the loss of CD127 concurrent with the gain of CD122, NK1.1, and some activating receptors such as NKG2D. Late iNK cells acquire increased expression of both activating and inhibiting receptors, and DX5. The final stage of development of iNKs into mature NK (mNK) cells is marked by the upregulation of CD11b and gradual loss of CD27 in mice. CD56 and CD16 provide similar role in humans [151]. Expression levels of these markers, in particular CD27 in mice, and CD56 in humans, denotes subsets with increased cytotoxic potential versus immunoregulatory functions [151,152].

Development of mature NK cells is dependent on both intrinsic (sequential activation of various transcription factors, such as T-bet and eomesdermin (Eomes), and micro RNAs which act as post-transcriptional repressors of messenger RNA (mRNA)), and extrinsic regulation by accessory cells [138,140,153]. These accessory cells include

dendritic cells, monocytes, and neutrophils [154-157]. Mature dendritic cells (DCs), or more recently, monocytes, can trans-present IL15 through the IL15Rα to its cognate receptors (IL15Rβ, and γ_c) on NK cells [158-161]. Unlike other ILCs, which require IL-7, NK cells are completely dependent upon IL15 for their development, activation, and survival [139,140,162,163]. In addition to IL15, DCs and monocytes also secrete NK-activating cytokines such as IL12 and IL18 [163,164]. Recently, neutrophils have been shown to have a non-redundant role in both the terminal differentiation of NK cells, as well as their maintenance and activation within tissues, in both humans and mice [155].

In addition to the activating role of various cytokines from accessory cellular sources, NK cells express various inhibitory and activating receptors whose binding during cell-to-cell contact set a threshold for NK cell activation, and potential cytolytic potential. Both immature, and mature NK cells are capable of directly killing stressed, transformed, infected, and under- or over-activated cells. Natural killer cells deliver death signals through a variety of mechanisms: perforin/granzyme release, death receptor ligation, antibody-dependent cellular cytotoxicity (ADCC), and cytokine release [165,166]. Due to their ability to kill without prior sensitization, the manner by which these cells become activated is tightly regulated; therefore, preventing the uncontrolled killing of normal, healthy cells. In order to achieve activation, NK cells typically require multiple activating signals, and an absence, or limited inhibitory stimulation via their respective receptors. However, this is not merely a balancing act as it has been shown that binding of inhibitory receptors is a more dominant interaction. Murine NK cells express a variety of activating receptors, such as natural cytotoxicity receptors (NCRs –NKp44,

NKp46, NKp30), c-type lectin superfamily receptors (Ly49- D, H, P, NKG2D), CD16, and DNAM-1. Human NK cells do not have Ly49 receptors, but instead express killer Ig-like receptors (KIRs), which like Ly49 receptors have both activating and inhibitory members. In addition to the inhibitory Ly49 receptors, NK cells also express inhibitory receptor, CD94/NKG2A. The number of activating and inhibiting receptors are not limited to those listed above, and new receptors are continually discovered, as are the ligands that bind them [166]. Mounting evidence has shown that NK cells can be tuned by their environment and undergo an educating or licensing process which allows them to gain or maintain a specific level of responsiveness to further activating signals. NK cells that are "uneducated" are rendered hyporesponsive. This state of licensing is not static, and can be altered based on length of exposure to specific environmental stimuli, and is highly dependent upon inhibitory signaling [167]. For instance, a critical inhibitory ligand for NK cells is MHC class I (MHC-I, major histocompatibility complex), and NK cells from MHC-I-deficient mice are found to be "uneducated" as they are impaired in both their responses to tumor cells, and stimulation of activating receptors. However, when NK cells from MHC-I-deficient mice were transferred into a wild type (WT) mouse background, they acquired increased reactivity, whereas WT NK cells that are transferred to mice lacking MHC-I revert to a hyporesponsive state [168-170].

In addition to tuning of NK responses towards a specific environment, mounting evidence has shown that NK cells can exhibit memory-like phenotypes, a phenomenon typically restricted to adaptive immune responses [171]. This memory-like phenotype was first observed in a model of chemical hapten-induced contact hypersensitivity (CHS)

in RAG2-/- mice, which lack typical adaptive immune cells (T and B cells) [172]. It was discovered the NK cell-mediated CHS responses could be detected up to a month after sensitization, and this effect was hapten-specific as only the hapten used to prime the mice could stimulate the memory-like response. Previous to this finding, it was thought only CD4+ T cells were responsible for hapten-memory responses. Since these initial observations of NK memory functions, a variety of studies have reported NK memory in virally infected animals, particularly with MCMV [173-175]. Indeed these memory-like functions are not limited to viral, or hapten-induced activation, but a recent study has shown that NK cells stimulated with cytokines *in vitro* and then transferred to naïve animals retain enhanced responsiveness and secretion of cytokines upon restimulation, even months after their initial stimulation and subsequent adoptive transfer [176]. These findings suggest that mature NK cells that become activated can then acquire an inheritable stable state, which can then enable robust, and accelerated responses to subsequent exposures. This state can be maintained through transfer to multiple naïve hosts, and offer protective effects to these animals.

In addition to exhibiting adaptive qualities of memory responses, NK cells can also shape adaptive responses through a variety of mechanisms, such as cytokine and chemokine production, cytolysis, and altering antigen presentation [177-180]. Aside from being major producers of IFNγ, NK cells can produce numerous other proinflammatory (TNFα) and anti-inflammatory cytokines (IL-10 and TGFβ), as well as growth factors (M-CSF and GM-CSF). Though the impact of growth factor secretion by NK cells is unknown, secretion of pro-, or anti-inflammatory cytokines can shape T cell responses

[181]. Secretion of these cytokines, as well as various chemokines (MCP-1, MIP1α, MIP1β, RANTES, and lymphotactin), can aid in directing the activation of a variety of other immune cells, and their homing to the site of inflammation, or from inflamed tissue to secondary lymphoid compartments. The latter, along with NK-expressed IFNγ has been shown to be crucial for generating Th1 responses. However, it is not only soluble factors produced by NK cells which direct T cell immunity, as direct cell-to-cell contact and receptor-ligand interactions (NK and DC, or NK and T cell) have been shown to activate, or limit DC antigen presentation, as well as CD4+ and CD8+ T cell responses [182-185]. For instance, NK cells can activate DCs, not only through cytolysis of target cells thus providing potential antigen for DC uptake, but also through secretion of IFNγ, which stimulate DCs to produce IL12 and IL15, therefore further increasing NK cell stimulation, and in turn further stimulating DC maturation [186]. Exposure of activated NK cells to IL12 can also trigger NK cell killing of immature DCs; therefore, selecting for mature DCs that are capable of triggering robust T cell responses towards invading pathogens [187,188].

NK cells and Citrobacter

While a role for NK cells in host defense for viral and intracellular bacteria has been well documented within the literature, a unique role for these innate immune cells in mediating immune responses to extracellular bacteria has only recently been investigated. Natural killer cell-depleted mice infected with *C. rodentium* exhibit diminished immune

cellular responses within the colon, in particular IFNγ-producing Th1 CD4+ T cells [189,190]. These studies found increased burden in depleted animals, yet there was discrepancy over whether the depletion of NK cells negatively or positively influenced intestinal pathology. Differences in these findings may be due to different methods of depletion used in these studies. However, a correlation of loss of IFNγ-producing NK cells, as well as diminished adaptive IFNγ+ CD4+ T cells, with greater intestinal pathology is similar to phenotypes previously reported for IFNγ-/- mice [90,136,189,190].

Macrophages

Macrophages represent a diverse branch of the innate immune system. Since their initial discovery as phagocytes, a greater appreciation has arisen for the ability of macrophages to respond to environmental cues, and adapt to these environments with a high degree of plasticity. Due to their plastic nature, these phagocytes play an essential role in a wide array of processes, including bacterial and tumor surveillance, development, homeostasis, metabolism, inflammation, and tissue repair [191-199]. Macrophages are typically classified as either classically activated (M1), or alternatively activated (M2, or M2-like) [200-202]. These designations were set to align with Th1 and Th2 T cells responses, which rely upon IFNγ, or IL4/IL13, respectively. Classically activated (M1) macrophages receive signals from IFNγ, as well TLR ligands, to induce expression of reactive nitrogen and oxygen intermediates, proinflammatory cytokines and chemokines, and increased bactericidal, or tumoricidal activity. These M1 macrophages

are also important for generation of Th1 responses. Alternatively activated macrophages respond to IL-4 and IL13 (M2), or IL10 (M2-like), and exhibit upregulation of scavenger, mannose, and galactose receptors, which is accompanied by an increased phagocytic capacity [191,203]. These alternatively activated macrophages have been found to play important roles in tissue remodeling, immunoregulation, and tumor progression [191]. Despite these classifications, in the absence of an active infection, macrophages in tissues rarely exhibit a singularly activated population, and are commonly found to exist in mixed populations that show activation states somewhere within the M1 and M2 spectrum [204,205].

Previously thought to be relatively short-lived immune cells, recent evidence has emerged to show that specific tissue resident macrophages have origins in fetal derived macrophage that develop prior to hematopoiesis [206-208]. These macrophages are long-lived, and persist into adulthood. Tissue derived macrophages play essential roles in homeostasis, tissue modulation, and surveillance [209]. The latter has been observed with macrophages actually patrolling along the endothelium in certain tissues [210]. Intestinal macrophages, unlike other tissue resident cells do not have fetal origin, but are constantly resupplied from blood monocyte precursors that develop within the bone marrow [211,212]. These monocytes, which express CX_3CR1, have the capacity to develop into either CD11c+ macrophages, or inflammatory dendritic cells depending on the environmental cues of the colon [213,214]. In the non-inflamed gut, CX_3CR1+ macrophages develop from these monocytes, and express IL-10. Though the production of this cytokine is important for maintenance of regulatory T cells (Treg) within the gut, it

is the ability of these cells to respond via IL10R (IL10 receptor) to Treg-produced IL10 that is critical for intestinal homeostasis [215,216]. A similar phenomenon is seen in humans with mutations in the IL10R gene, who have aggressive, and early onset of inflammatory bowel disease (IBD) [217]. The reliance upon IL-10 signaling to maintain their tolerogenic state within the intestine, and flip from tolerogenic to inflammatory in the absence of IL-10R signaling, show how plastic even a terminally differentiated macrophage population can be, and how their given designated activation state is reliant upon environmental cues.

Macrophages and Citrobacter

The ability of macrophages to display plasticity in their activation state is critical in pathogen defense, whether M1 for bacterial pathogens, or M2 for parasites defense. The importance of a particular designation is observed in mixed parasite and bacterial infections. Infection of helminth-infected mice with *C. rodentium* results in a parasite-directed Th2 environment within the colon, and a dysregulated Th1 response [218]. Loss of effective Th1 responses results in increased tissue damage, and morbidity and mortality in infected animals. This skewing of immune responses to Th2/M2 also impairs macrophage-directed killing of *C. rodentium* [218,219]. Depletion, or loss of macrophages in *Citrobacter*-infected mice results in increased burden, intestinal pathology, as well as diminished Th1 responses [220,221].

Th1 CD4+ T cells

Naive CD4+ T cells differentiate within the draining lymph node (in the case of the gut, the mesenteric lymph nodes) into one of several distinct T helper type (Th) T cells: Th1, Th2, Th17, and induced Treg [222]. Activation of naive T cells requires not only recognition of MHC-foreign peptide complexes presented by antigen presenting cells (APCs), in particular DCs, but also co-stimulatory molecules, CD80/86 and CD40 [223,224]. Only T cells whose T cell receptor (TCR) recognizes and can bind to the foreign peptide presented by the APC, and receives secondary signals from costimulatory molecules, will proceed with initiation of activation, and eventual differentiation, and clonal amplification [225]. Unlike CD8+ T cells, CD4+ T cells require persistent antigenic stimulation for complete differentiation into the T helper (Th1) effector phenotype [226,227]. Each lineage of CD4+ helper T cells has a unique repertoire of expressed cytokines, cell surface markers, and transcription factors critical for their differentiation. T helper cell type 1 (Th1) T cells rely on transcription factors T-bet, and Eomes for their expression of IFNγ, as CD4+ T cells lacking both of these transcription factors are incapable of expressing this cytokine. Cytokine cues are also critical for Th differentiation; for Th1 these include IFNα, -β, and -γ, and IL12 [228,229]. In particular, it is IL12 production by DCs, and NK-produced IFNγ that are essential for directing the generation of Th1 CD4+ T cells [229-231].

CD4+ T cells are capable of orchestrating multiple functions within the host: induction of B cell antibody responses, enhancing/regulating/suppressing immune

responses for which they control, as well as act as important mediators of memory functions of the adaptive immune system [222]. The importance of these cells in host defense to bacterial pathogens is exhibited in the increased susceptibility of HIV patients with diminished CD4+ T cells numbers to a variety of infectious diseases, in particular mycobacterial infections [232]. It is the loss of Th1 responses in these patients that typically mediates this increased susceptibility to mycobacterial infections [233]. While the necessity of Th1 CD4+ T cells for host protection from intracellular bacterial pathogens, like *Mycobacterium*, is well known; there is mounting evidence for an important role for Th1 responses in host defense strategies against extracellular bacterial pathogens.

Th1 responses and Citrobacter

A robust Th1 response has long been recognized as a hallmark of *Citrobacter rodentium* infections [72,234]. In general, CD4+ T cells play an essential role in *C. rodentium* defense, as mice depleted of CD4+ T cells, or Rag1-/- mice (lacking T and B cells) succumb to infection [235,236]. It was later determined that host survival was dependent upon the expression of co-stimulatory molecules, CD28 and CD40L, which play essential roles in CD4+ T cell activation, and differentiation [237]. Th1 CD4+ T cells responses were not found to independently have an effect on host survival in wild type mice; however, mice lacking IFNγ-producing CD4+ T cells exhibited increased bacterial burden, delayed clearance, and increased intestinal pathology similar to those

observed in IFNγ-/- mice [90,136,237]. Additionally, Th1 CD4+ T cell responses have been shown to be critical for Ig class switching, and production of IgG2a (IgG2c in C57BL/6 mice) pathogen-specific antibodies [237]. In *Citrobacter* infections, loss, or delay of Th1 responses, like what is observed in NK cell-depleted mice, results in diminished pathogen-specific IgG responses, which correlated with increased systemic bacterial burden [189,190,238]. Adoptive transfer of immune sera into B cell-deficient mice revealed that IgG is important for clearance of *Citrobacter* from systemic sites [236,239]. It has also been speculated that IFNγ produced by Th1 CD4+ T cells at later time points help mediate macrophage phagocytosis of *Citrobacter* [136]. Beyond mediating IgG-directed responses and macrophage phagocytosis, Th1 CD4+ T cells were revealed to be the main cell type responsible for goblet cell depletion in the gut, as well as epithelial proliferation [70].

Goals of Study

EPEC infections are the second leading cause of diarrheal-related death in children [14,15]. With a high rate of mortality, and resistance to typical interventions, new potential therapeutics, as well as a greater understanding of host defense strategies directed against this pathogen are required [2,14]. *C. rodentium*, a natural murine pathogen, provides an excellent model for EPEC infection. Host responses to *Citrobacter* are characterized by a robust Th1 response [72,234]. At the time of commencement of these studies, the importance of IFNγ in controlling pathogen burden, clearance, and

intestinal pathology had been observed [90]. However, the cell types, other than Th1 CD4+ T cells, necessary for protective IFNγ-directed host responses were unknown. Natural killer cells had been shown to play an essential role in directing Th1 responses to both parasite and bacterial pathogen infections at various sites within the host, other than the gut [240-243]. Yet it was unclear if NK cells played a similar role for host protection against extracellular enteric pathogens. In addition to a potential role for NK cells in directing Th1 adaptive response, depletion of these cells in Rag1-/- (lacking T and B cells) mice led to increased susceptibility to infection, with NK-depleted animals succumbing to infection at a faster rate compared to controls [143]. Thus it was evident that NK cells were potentially mediating both innate and adaptive immune responses within host defense to *C. rodentium*. Therefore, the major hypothesis of this study was that **interferon γ- producing natural killer cells mediate both innate and adaptive responses to host pathogen, *Citrobacter rodentium***. The overall aims of this study were to: 1) Determine if NK cells are important for induction of Th1 responses in *Citrobacter rodentium* infection; 2) Determine if protective responses of NK cells, independent of an adaptive immune response, are mediated through IFNγ-dependent induction of CXCL9, an antimicrobial chemokine.

Chapter Two

$CD3^{-}NK1.1^{+}$ cells aid in the early induction of a Th1 response to an attaching and effacing enteric pathogen

Chapter Two – Co-authorship Statement

Chapter Two consists of the following publication:

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The following work was conducted by authors other than myself:

1) Analysis of immune cells populations in H&E histochemistry was performed by C.L.N.S.
2) Manuscript was written and edited by S.A.R-Y and B.K.C.

***NOTE: All references to IFNγ-producing lymphoid tissue inducer cells (LTis) within the published manuscript have been changed to innate lymphoid cells type 1 (ILC1s) within this Chapter as this has now become the common nomenclature.

$CD3^{-}NK1.1^{+}$ cells aid in the early induction of a Th 1 response to an attaching and effacing enteric pathogen

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Summary

Extracellular attaching and effacing (A/E) pathogens including pathogenic *E. coli* colonize the host gut causing diarrhea and inflammation. Although much is known regarding the pathogenesis of A/E bacteria, there remains an incomplete understanding of host immune responses to these microbes. Natural killer (NK) cells are an important source of IFN-γ and are essential for early innate responses to viral pathogens, however their role during extracellular bacterial infections is still largely unexplored. We studied the host response to the murine A/E pathogen *Citrobacter rodentium* to investigate NK-cell function during infection. $NK1.1^+$ cell depletions and analysis of colonic intestinal inflammation following *Citrobacter* infection demonstrated that $CD3^-NK1.1^+$ cells play an important role in the initial clearance of *C. rodentium*, as evidenced by higher bacterial load, intestinal pathology, and crypt hyperplasia at the peak of inflammation in depleted mice. Loss of $CD3^-NK1.1^+$ cells resulted in lower colonic IFN-γ, TNF-α, and IL-12, and a delay in homing of IFN-γ^+ $CD4^+$ T cells to the gut. Loss of this response resulted in lower anti-*C. rodentium* IgG in NK1.1-depleted mice. These data establish that $CD3^-NK1.1^+$ cells are critical for inducing an early Th1 response involved in clearance of a pathogen that is restricted to the gastrointestinal tract.

Introduction

Attaching and effacing (A/E) pathogens are important causative agents of intestinal disease worldwide. A/E pathogens, such as enteropathogenic *E. coli* (EPEC), cause severe diarrhea, vomiting and fever with high rates of fatality especially in young children and the elderly in developing nations [244]. Like other A/E pathogens, EPEC attaches to the epithelium of the colon through pedestal-like formations involving effacement of the colonic microvilli. This attachment is essential for virulence of the organism [245-247]. The majority of research concerning A/E pathogens has focused on how these intimate contacts with the intestinal wall are formed and contribute to disease outcome. However, recent work has shown that a significant portion of the symptoms and pathology associated with the disease are a direct result of the host immune responses towards these pathogens. This has been facilitated by the study of *Citrobacter rodentium*, a natural murine A/E pathogen, to model human EPEC infections in mice. Both EPEC and *C. rodentium* contain the locus of enterocyte effacement (LEE) pathogenicity island [248] encoding a type III secretion system common to many Gram-negative pathogens [25,249,250].

A typical *C. rodentium* infection in mouse strains that do not succumb to infection results in colonization of the cecum during early stages of infection after which the bacteria colonize mainly the distal colon. Bacterial numbers reach a peak at approximately one week post infection with the inflammatory response reaching a peak by two weeks coinciding with the initiation of clearance. Bacterial clearance continues

through weeks 3 and 4 [26]. Host responses necessary for pathogen clearance involve $CD4^+$ T cells, B cells, mast cells, and neutrophils [235,239,251-253]. IgG responses are also required, whereas IgA and IgM responses are not essential [238,239]. The infection leads to elevated T helper cell 1 (Th1) and Th17 responses, and mice lacking the cytokines needed for these responses (interferon gamma (IFN-γ), and interleukin 12 (IL-12) and IL-17, and IL-22 respectively) have increased susceptibility to *C. rodentium* [254-256]. Recently it was determined that the IFN-γ produced by $CD4^+$ T cells is essential for controlling pathology, bacterial load, as well as clearance of the infection [136]. However, the signals necessary for initiating this response have not been examined. In 2004, the early stimulation of natural killer (NK) cells and their trafficking to the draining lymph node was found to be essential for early activation of the $CD4^+$ Th1 response [231]. Recent work in various parasite infection models [240,242,257] and the obligate intracellular bacterium *Chlamydia muridarum* [241] showed that IFN-γ produced by NK cells during initial stages of infection is critical for directing an early Th1 response towards these organisms. However it is currently unknown how Th1 responses are initiated by extracellular pathogens localized to the gastrointestinal tract.

Natural killer cells are known to play a key role in controlling viral infections such as MCMV due to their ability to kill without prior sensitization [258]. However their role in defense against bacterial pathogens has only just begun to be investigated. Recent work by Hall et al. showed in an asialo-Gm1 depletion model of *Citrobacter rodentium* infection, a dependence on NK cells for inducing a robust inflammatory response, including CD3+ cell homing to the colon [189]. Here we investigated the role of NK1.1+

cells in a *C. rodentium* infection model. We find that depletion of $NK1.1^+$ cells results in higher bacterial numbers and pathology in the colon at the peak of inflammation, which is accompanied by lower levels of Th1 cytokines, *Citrobacter*-specific IgG, and delayed homing of IFN-γ^+ $CD4^+$ T cells to the site of infection. Our data report a new role for $CD3^-NK1.1^+$ cells in stimulating an early Th1 response and homing of $CD4^+$ IFN-γ^+ T cells to the gut in response to an enteric pathogen restricted to the gastrointestinal tract.

Results

C. rodentium infection induces NK-cell activation and rapid trafficking to MLN.

We first examined the dynamics of the NK-cell response to *C. rodentium* infection. Higher numbers of the activated $CD3^-NK1.1^+$ cell population was observed in the MLN two days post infection (**Fig. 1A-C**). Lymph nodes are typically devoid of NK cells, however their presence at these sites at early time points is important for stimulating a Th1 response [231,259]. Higher numbers of $CD3^-NK1.1^+$ cells in the MLNs was transient and did not appear at any other time point examined (**Fig 1B**). Next we examined activation of NK cells at the site of infection and found higher numbers of IFN-γ-expressing $CD3^-NK1.1^+$ cells in the spleen, and colonic lamina propria (**Fig. 1D**) at day 2 after infection when compared with that of uninfected controls. This peak of NK-like cell activation diminished in the spleen at later time points, although the total number of activated $CD3^-NK1.1^+$ cells was still significantly higher compared to levels in uninfected control mice. In the colonic lamina propria there is an additional peak of $CD3^-NK1.1^+$ response at day 10 after infection in addition to day 2. As NK1.1 is expressed on several immune populations other than NK cells, such as NK-T cells, and CD8+ T cells, we examined the number of $CD3^+NK1.1^+$ cells producing IFN-γ at various time points in the colonic lamina propria and did not observe any increase in number or activation of this population (Supporting Information **Figure 1 A)**. Recently, it was observed that specific $CD3^-NK1.1^+$ NK-like innate lymphoid cell type 1 (ILC1) cells in the intestine are capable of downregulating RORγt, and upregulating expression of IFN-γ [260]. We analyzed the responding $CD3^-NK1.1^+$IFN-γ^+ population at day 2 post infection for ILC1 marker

CD127, and found that approximately 15% were positive (Supporting Information **Figure 1 B**). This appears to be a ~5% increase compared with uninfected controls, however, the majority of responding $CD3^{-}NK1.1^{+}$ cells appears to be conventional NK cells. These data suggest that both conventional NK cells, and NK-like ILC1s, but not other $NK1.1^{+}$ immune populations, are activated rapidly upon infection with *C. rodentium* and are likely key sources of IFN-γ at early and intermediate time points at the site of infection.

Loss of NK cells results in greater pathology and higher bacterial loads.

To assess the role of these $CD3^{-}NK1.1^{+}$ cells during *C. rodentium* infection, we treated mice with anti-NK1.1 antibody. Effective depletion of $NK1.1^{+}$ populations was assessed at several time points and in various tissues (Supporting Information **Figure 2**). Upon examining the fecal load of *Citrobacter*, it appeared that there was no difference in the number of bacteria being shed by the NK1.1-depleted mice and PBS-treated control mice at early time points of infection (days 4, 6, and 7) **(Fig. 2A**). We also observed similar bacterial burden in the colon at day 11. In contrast, at the peak of inflammation (day 14), we found maintenance of high colonization levels of *C. rodentium* in the colon of NK1.1-depleted mice; whereas PBS treated mice had commenced initial clearance of the infection (**Fig. 2B**). We also observed higher numbers of systemic bacteria in the spleens of NK1.1-depleted mice (**Fig. 2C**) suggesting a clearance defect. Pathology scores were similar on day 11 after infection between the groups of mice (**Fig. 2D and 2E**), however higher bacterial load at day 14 coincided with increased desquamation of the epithelial layers (**Fig. 2D and 2E**) and greater crypt hyperplasia in the distal colon

(**Fig. 2F**). Use of the NK1.1 antibody did not have an independent effect on pathology since long-term depletion of $NK1.1^+$ cells caused no differences when compared to uninfected mice. These data suggest that $CD3^-NK1.1^+$ cells play a role in early clearance of *Citrobacter*, as well as protect against pathology associated with the infection.

Depletion of NK cells results in diminished Th1 cytokine and chemokine responses.

NK cells are an important source of IFN-γ. Previous work showed that loss of IFN-γ resulted in increased pathology during *C. rodentium* infections [136,254]. Through their interactions with other immune cells, typically dendritic cells (DCs), NK cells also increase levels of other cytokines such as interleukin 12 (IL-12) and tumor necrosis factor alpha (TNF-α) [261]. To examine the levels of these cytokines, we excised and cultured infected colons of mice and analyzed the supernatants using ELISA. In the colons of NK1.1-depleted mice we observed significantly lower levels of IFN-γ compared to PBS treated controls at both day 6 and 14 post infection (**Fig. 3A**). Interleukin 12 levels were also significantly lower at day 14. The levels of TNFα were not significantly lower than controls at either time point, however, there was a trend for lower levels of this cytokine in the colons of depleted mice. These results were comparable with transcript levels observed for each of these cytokines (data not shown). We also examined IL-17, IL-22, and iNOS2 levels and found no differences in the transcript levels between groups at this time point (data not shown). Previous studies have shown that the loss of Th1 cytokines results in increased damage to the epithelium during *C. rodentium* infections [254]. Thus the loss of $NK1.1^+$ cells and lower levels of Th1 cytokines in the gut likely contribute to

the higher susceptibility to *C. rodentium* infection and the corresponding increase in pathology.

Early IFN-γ responses are important for inducing chemokine secretion, which is essential for immune cell homing to secondary lymphoid tissue as well as to the site of infection [262]. In particular, IFN-γ promotes chemokines CXCL9, 10, and 11 [101]. We measured CXCL9/MIG protein levels in the distal colon of infected mice at day 6 and day 14 and found lower levels of the chemokine at both day 6 and day 14 in NK1.1-depleted mice compared with those of controls; however, this trend did not reach statistical significance **(Fig. 3B).** These data suggest that loss of $NK1.1^+$ cells results in a trend toward lower levels of IFN-γ-induced chemokines. Taken together, we find that depletion of $NK1.1^+$ cells causes a diminished host response due to lower levels of Th1 cytokines and chemokines at both early time points and at the peak of inflammation.

Loss of NK cells delays the Th1 $CD4^+$ T-cell response.

Downgraded Th1 responses and decreased activation of $CD4^+$ T cells typically results in decreased IgG responses during infection. Multiple studies have examined the importance of IgG responses in *C. rodentium* clearance [238,239]. The antibody response stimulated by $CD4^+$ T cells is the main role of $CD4^+$ T cells during *C. rodentium* infection since dosing CD4-depleted mice with wild type serum prevents infected mice from succumbing to the infection [238]. To examine whether loss of $NK1.1^+$ cells affected adaptive responses during *C. rodentium* infection, we tested plasma IgG responses to the

pathogen. We examined *Citrobacter*-specific total IgG levels in plasma of mice at various time points post infection using an ELISA. Significantly lower levels were observed in depleted mice compared with those of controls at day 14 (**Fig. 4**), implying that NK1.1-depleted mice are not capable of generating an adaptive response typically observed at day 14 post infection.

Multiple studies have shown a key role for NK cells in stimulating a Th1 response during bacterial infection [240,241]. The higher susceptibility to infection combined with lower inflammatory cytokine and chemokine responses suggested a delayed Th1 response in NK1.1 depleted mice. To address whether $CD4^+$ T cells were dampened in NK1.1-depleted mice we stained for $CD4^+$ cells in the distal colon and found lower numbers of $CD4^+$ immune cells in NK1.1-depleted mice compared to PBS controls at day 14 (**Fig. 5A-B**). Typically, *C. rodentium* infection causes massive infiltration of $CD4^+$ T cells, particularly IFN-γ^+ populations [234]. To confirm whether the decreased Th1 cytokine response we observe at day 14 is due to an absence of this immune population, we isolated lymphocytes from the colonic lamina propria at various time points during infection and examined total cell numbers of $CD4^+$ IFN-γ^+ T cells. Gating on $CD49^+CD3^+$ lymphocytes, there was comparable numbers during early stages of infection of $CD4^+$IFN-γ^+ T cells in the gut, however at day 14 this was higher in the control group compared with that in NK1.1-depleted mice (**Fig. 5C-D**). As memory $CD4^+$ T cells can also express the NK1.1 marker, we wanted to determine if the phenotype observed in depleted mice was due to depletion of this cell population. We examined expression

levels of NK1.1 on the $CD4^+$ IFN-γ^+ T-cell population present at day 14; however, there was no appreciable expression of the NK1.1 marker within this population (Supporting Information **Figure 1**). Therefore, we conclude that it is the loss of $NK1.1^+$ cells, and not direct antibody-mediated $CD4^+$ T-cell depletion that results in a delayed homing of $CD4^+$IFN-γ^+ T cells to the colons of depleted mice as well as a delayed IgG response to the pathogen.

Loss of NK cells can be compensated for at later time points of infection.

Previous work examining the role of IFN-γ during *Citrobacter* infection found that not only was pathology increased in IFN-$\gamma^{-/-}$ mice, there was also a delay in bacterial clearance [136]. We investigated whether the delayed Th1 response in NK1.1-depleted mice results in a long-term delay in bacterial clearance. When we examined bacterial burden in the colon at days 18 and 21, we found no significant difference between groups in bacterial levels in the colons of NK depleted mice compared to controls (**Fig. 6A**), however, there was slightly greater pathology at day 18 in depleted mice (**Fig. 6B-6D)**. We had also examined IgG responses at day 18, and found them to be equivalent between groups (data not shown) indicating that the $CD4^+$ Th1 response is delayed and not absent in these animals. We therefore examined IFN-γ^+CD4^+ T cells numbers at this time point and found that $CD4^+$ T-cell numbers were similar between depleted mice and controls (**Fig. 6E-F**). These data suggest that while there appears to be an initial delay in clearance of *C. rodentium* during the peak of inflammation, recovery of the IgG response and Th1 response by day 18 corrects for this delayed clearance phenotype.

Early delay in bacterial clearance is not observed in IFN-$\gamma^{-/-}$ mice depleted of NK cells.

As NK cells are important sources of IFN-γ, which is a critical factor in stimulating a Th1 response, we investigated whether this was the primary mechanism by which the NK1.1^{+} cells are acting during *C. rodentium* infection. We depleted NK1.1^{+} cells in IFN-$\gamma^{-/-}$ mice and examined bacterial burden at day 14. In the colon and spleen of both the NK1.1-depleted mice and PBS-treated mice we found that the absence of IFN-γ did not affect bacterial burden at this time point (**Fig. 7A-B**). This implies that the primary role of the NK1.1^{+} cells during *C. rodentium* infection is to supply an early source of IFN-γ in order to stimulate a timely Th1 response to control both bacterial numbers and pathology in the distal colon. This establishes an important role for this host defense mechanism against a strictly extracellular bacterial pathogen localized to the gastrointestinal tract.

Discussion

Previous work has established a critical role for NK cells in stimulating Th1 responses to intracellular bacteria and parasitic infections [240,241]. However, it was unclear whether a similar role for NK cells would be observed in extracellular infections restricted to the gastrointestinal tract. The data presented here support the hypothesis that $CD3^-NK1.1^+$ cells are important for directing early Th1 responses to the enteric pathogen *C. rodentium*. In this study, we observed higher numbers of IFN-γ-producing $CD3^-$ $NK1.1^+$ cells in colonic lamina propria in response to *Citrobacter* infection. To rule out a potential role for NK-T cells contributing to early IFN-γ production, we examined the total numbers of $CD3^+NK1.1^+$ immune cells in the colon, but did not observe higher cell numbers, or increased IFN-γ production. However, we did observe that a proportion of the $CD3^-NK1.1^+$ cells were positive for CD127, leading us to conclude that NK-like ILC1s in addition to conventional NK cells are capable of responding to infection. Therefore, we attribute the effects observed in these studies to an IFN-$\gamma^+CD3^-NK1.1^+$ immune population.

A recent study by Hall et al. examined the role of asialo $Gm1^+$ NK cells in *Citrobacter* infections and found an overall dampened immune response in the absence of NK cells including a decreased number of $CD3^+$ cells at day 14 post infection [189]. Interestingly, they observed a lack of pathology in their depleted animals, contrary to our findings of greater pathology due to NK cell depletion. This discrepancy is likely due to the different mechanisms of depletion. Anti-asialo Gm1 depletes conventional NK cells

but has also been reported to deplete certain populations of activated macrophages [263]. In addition, as discussed above, the NK1.1 depletion used in our studies depleted NK-like ILC1s in addition to conventional NK cells; it is unknown what effects this NK-like population has in determining pathology outcomes in *Citrobacter*-driven colitis. Recently, it has been observed that depletion of IFN-γ-expressing ILCs dampens *Salmonella*-directed enterocolitis [264]. The *Salmonella* colitis model has pathology that is dependent on increased IFN-γ [265]. *Citrobacter*-infected mice do not appear to follow this paradigm as IFN-$\gamma^{-/-}$ mice suffer from increased levels of pathology [254] and loss of IFN-γ^{+}CD4^{+} T cell responses appear to play an important role in limiting intestinal damage [136]. Therefore, the increased pathology observed in our model agrees with the role of IFN-γ^{+}CD4^{+} T cells in controlling pathology, however, CD3^{-}NK1.1^{+}IFN-γ^{+} cells may also play an important role in limiting pathology in an IFN-γ-dependent manner.

The importance of IFN-γ production by NK cells in the homing of CD4^{+} T cells to sites of infection has been reported for both adjuvant-stimulation models as well as bacterial infections [231,240,241]. Initial work in a murine footpad LPS-stimulation model showed that NK cells trafficked to draining lymph nodes in a CXCR3-dependent manner, providing critical early sources of IFN-γ essential for generating a Th1 response [231]. Our data shows CD3^{-}NK1.1^{+} cells trafficking to the mesenteric lymph nodes at day 2 post infection suggesting these cells play a key role in stimulating a Th1 response. We also observed lower levels of the CXCR3-binding chemokine, CXCL9, in NK1.1-depleted mice at both day 6 and day 14. Elevated CXCR3-binding chemokine levels likely allows NK cells to traffic to the MLNs during a typical infection, as well as

CXCR3-dependent homing of $CD4^+$ T cells to the lymph node and the site of infection. Although the observed differences between NK1.1-depleted mice and PBS controls do not reach significance, the trend toward lower levels of IFN-γ-induced chemokines at the peak of the inflammatory response could potentially affect homing of the Th1 T-cell population to the site of infection. Further work is required in order to tease apart the separate roles of NK cell trafficking to the lymph node and lower chemokine responses in controlling the homing of IFN-γ^+CD4^+ T cells to the infected colon.

Previous studies provided evidence that the IFN-γ-dependent crosstalk between NK cells and DCs is essential for activation of $CD4^+$ T cells and initiation of a Th1 response [231]. A similar role for NK cell-derived IFN-γ and its stimulation of DCs in driving a pathogen-specific Th1 response was also observed in several bacterial and parasite infection models [241,242,257]. Loss of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine critical for maturation of DCs from activated monocytes, resulted in increased susceptibility to *C. rodentium* infection [266]. Loss of $CD11c^+$ DCs resulted in lower IgG serum levels as well as increased pathology in the gut. While DCs may play additional roles in clearance of *Citrobacter*, their early stimulation of NK cells - and by NK cells - likely plays a critical role in initiating the Th1 response to *C. rodentium*. We observed lower levels of IL-12 at day 14 post infection in NK1.1-depleted mice, likely a result of a delayed DC activation. This connection between NK cells and DCs in stimulating the Th1 responses in our model is a topic currently under investigation.

IgG responses, but not IgM or IgA, are important for clearance of *Citrobacter* infection [238,239]. Mice lacking $CD4^+$ T cells are highly susceptible and succumb to infection, however serum from wild type infected mice protects them following infection [238]. We found that the loss of $NK1.1^+$ cells resulted in a delayed IgG response. Serum IgG aids in decreasing systemic bacterial levels caused by passive flow of bacteria from the damaged epithelium of the gut. Lower IgG levels observed in this study likely allowed for the higher levels of *Citrobacter* observed in the spleen of mice lacking NK cells. The recovery of IFN-γ^+CD4^+ T cells at later time points also results in IgG levels equivalent to wild type, and corresponds with a recovery of the adaptive response.

The recovery of the Th1 response at later stages of infection in NK1.1-depleted mice implies a possible secondary source of IFN-γ capable of stimulating a Th1 response. While identifying additional innate sources of IFN-γ in our model is of importance, it has been reported that in the absence of NK cells, an endogenous IFN-γ response from $CD4^+$ T cells and IL-12 expression from DCs is sufficient for generating a Th1 response [267,268]. However, this phenomenon may be restricted to Th1-prone strains of mice, such as C57BL/6, and further experimentation is necessary to determine which is responsible for compensating the loss of NK cells observed at later time points [269].

In conclusion, our data show that loss of $NK1.1^+$ cells during *C. rodentium* infection is critical for early stimulation of the Th1 response to the pathogen. The overall effect of the depletion of $NK1.1^+$ cells results in poor initial clearance of the bacteria from the gut. Secondary sources of IFN-γ, or use of a Th1-prone mouse strain, seem to compensate this loss during later stages of infection resulting in wild type levels of

$CD4^+IFN\text{-}\gamma^+$ T cells, but not before increased damage to the intestine has occurred. This work is the first to describe this role for $CD3^-NK1.1^+$ cells in response to a strictly enteric bacterial infection and confirms an important role for $IFN\text{-}\gamma^+CD4^+$ T cells in host defense against A/E pathogens.

Materials and Methods

Ethics Statement. All experiments with animals were conducted according to guidelines set by the Canadian Council on Animal Care. The local animal ethics committee, the Animal Review Ethics Board at McMaster University, approved all protocols developed for this work.

Mice. Animals were housed in a specific pathogen-free unit in the Central Animal Facility at McMaster University. All experiments were approved by and performed in accordance with the McMaster Animal Research Ethics Board. Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River. Female IFN-$\gamma^{-/-}$ mice (6-8 weeks old) were purchased from The Jackson Laboratory.

***Bacterial infections*.** Mice were orally gavaged with $2x10^8$ colony forming units (cfu) of *Citrobacter rodentium* DBS100 in 0.1 mL phosphate buffered saline (PBS). At specified time points, mice were euthanized and their organs were harvested. The spleen and colons were placed into 1 mL PBS on ice and then homogenized (Mixer Mill 400; Retsch). Organ homogenates were serially diluted in PBS and plated on Brilliant Green agar (EMD and Oxoid) for bacterial enumeration. Limit of detection for cfu estimation in the colon was 10^{-3}, and the spleen was 10^{-1} as previously determined [239]. All samples below the detection limit were assigned a value of 5 cfu for statistical metrics. Separate sections of the mesenteric lymph nodes (MLN), spleen, and colon were processed for RNA isolation, and histology.

NK1.1 depletion. Prior to infection, mice were injected intraperitoneally with 200 µg anti-mouse NK1.1 antibody (PK136 mouse IgG2a hybridoma HB191; ATCC). Mice were injected on two consecutive days 48 h prior to infection with *C. rodentium*. Throughout experiment, mice were injected with either antibody or control PBS every 3 days.

Isolation of lamina propria

Infected mice were euthanized at specified time points and entire colon was harvested and placed on ice in PBS. Colons were emptied of fecal content and thoroughly washed with PBS prior to cutting each colon longitudinally, and then into 3- to 8-mm sections. Colon pieces were washed four times in PBS, 5% FCS, 5mM EDTA for 20 min at 37°C while shaking at 240 rpm in order to remove the epithelial cell layer. After each wash, colon pieces were vortexed for 15 s, then collected in a 100 µm cell strainer, and placed into fresh buffer. Once buffer remained clear from debris after shaking, colon sections were placed into 10 mL digestion media (IMEM media (Gibco), 1% HEPES, 6mg DNase I (Sigma), 5 mg collagenase VIII (Sigma)) and shaken at 240 rpm at 37°C for 40 min. After 20 min, samples were filtered through a 40 µm cell strainer, and remaining undigested sample was placed in fresh digestion media and incubated for a further 20 min. Flow through from both incubations were combined, and washed with RPMI media (Gibco). Resulting pellet was resuspended and layered over an osmotic 100%/30% Percoll gradient, and then centrifuged at 670 *g* for 30 min at room temperature with no braking. Cells at the 100%-30% interface were collected and washed in RPMI. Cells were

then enumerated and plated at $1x10^6$ cells/mL in stimulation media, and incubated at 37°C, 5% CO_2 for 4 h.

Activation of Isolated Immune Cells

Isolated spleen and MLNs from euthanized mice were scraped on 40 μm cell strainers to create single cell suspensions. Cells were enumerated and then plated at $1x10^6$ cells/mL in stimulation media: RPMI, 10% FCS, 1% Penn/Strep, 1% HEPES, 1% Non-essential amino acids, 1% L-glut (Gibco) with 5 ng/mL PMA (Sigma) and 1 μM ionomycin (Sigma). GogliPlug (BD Biosciences) was added to each sample according to the manufacturer's protocol. Samples from the MLNs, spleen, and lamina propria were stimulated for 4 h prior to immune staining.

Murine histopathology. Upon euthanization, 30 mm sections of the distal colon was placed in 4% PFA (diluted from 16% PFA in PBS) for 72 h then transferred to 70% ethanol. Fixed colons were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). For pathological scoring, 10 fields were examined and scored as previously described [270-272]. In brief, crypt heights were measured using Image Pro software (Media Cybernetics) on an Olympus BX51 microscope with ten well-oriented crypts measured per field. Intestinal inflammation was quantitatively analyzed by observing submucosal edema, polymorphonuclear cell infiltration, epithelial integrity, and goblet cell depletion (Table S1). Pathology scores were given arbitrary units with a range from 0 to 13: 0 = intestine intact with no signs of inflammation; 1-2 = minimal

signs of inflammation; 3-4 = slight inflammation; 5-8 = moderate inflammation; 9-13 = severe inflammation. For immunohistochemistry staining, colons were embedded in OCT (Fisher), and rapidly frozen in liquid nitrogen. Colons were stored at -80 C prior to sectioning. Serial 5 μm sections were cut and fixed in 75% (v/v) acetone/25% (v/v) absolute ethanol solution for 5 min. Tissue sections were blocked with Rodent Block M (Biocare Medical) and then incubated with rat anti-mouse CD4 antibody (dilution 1:50) (PharMingen). After multiple washes with Tris buffered saline (TBS), slides were probed using a Rat on Mouse Kit (Biocare Medical) according to manufacturer's specifications.

ELISAs. At specified end points, colons of infected mice were harvested, flushed with sterile PBS, opened longitudinally, and cut into 5-10mm pieces. Colons were then placed into 1 mL complete RPMI containing 1%Penn/Strep/Gentamycin and incubated at 37°C. After 24 h, media was removed and centrifuged at 10 000 rpm for 3 min. Supernatants were removed and stored at -80°C. Cytokine and chemokine analysis was performed on supernatants using the following ELISA kits: Duoset murine IFN-γ (R&D Systems), Duoset murine TNFα (R&D Systems), and Duoset murine CXCL9/MIG (R&D Systems). All ELISAs were performed according to the manufacturer's protocols. Plates were read at 450 nm using a SAFIRE plate reader.

IgG ELISA. At the determined endpoint of the experiment, mice were anesthetized and euthanized by cardiac puncture exsanguination. Blood samples were immediately mixed to a 1:6 dilution with ACD anticoagulant, centrifuged, and plasma collected for total IgG

level determination. IgG ELISAs were performed by coating high-binding 96-well plates with heat-killed *C. rodentium* overnight at 4°C. Plates were washed three times with PBS, 0.05% Tween-20 prior to blocking for 1 h with PBS, 10% (v/v) soy milk, 0.05% Tween-20 at 37°C. Plates were again washed three times before addition of 100 µL serially diluted serum. Samples were incubated at 37°C for 2 h, washed, and then bound antibody was detected by incubating at room temperature for 1 h with polyclonal goat anti-mouse IgG antibody conjugated to HRP (1:10,000) (GE Healthcare). Washed plates were developed with 100 µL solution A and B (50%/50% mixture) (R&D Systems) for 15 min at room temperature, then reaction was stopped using 50 µL 1N H_2SO_4. Plates were read at 450 nm using a SAFIRE plate reader.

FACS. Stimulated cells were scraped off of the bottom of the well and centrifuged. Pellets were resuspended in 200 µL and 100 µL was plated per well (~$1x10^6$ cells) in round bottom 96 well plates. The plate containing the samples was centrifuged and the pellets resuspended in FACS buffer (PBS, 0.2% BSA). Cells were first incubated with 1/100 of anti-CD16/CD32 (eBiosciences) antibody for 15 min on ice, then incubated with 1/400 of anti-CD45-Alexa700, anti-CD3-FiTC (eBiosciences), 1/200 of anti-CD4-PerCP (BD Biosciences), 1/200 anti-NK1.1-PE antibodies (BD Biosciences) or isotype control antibodies for 30 min on ice. For intracellular staining, cells were fixed and permeabilized using GolgiPlug Kit (BD Biosciences) reagents. Cells were then stained with 1/200 anti-IFN-γ-APC antibody or isotype control for 30 min on ice prior to washing twice with permeabilization solution and suspension in FACS buffer. All samples were run on a

LSRII flow cytometer (BD Biosciences), and analyzed using FlowJo software (Tree Star, Inc). Staining for NK-like ILC1 populations included the following antibodies: anti-CD3 PE-Texas Red (BD Biosciences), anti-NK1.1 PE, anti-CD127-Alexa 488 (eBiosciences), or isotype control antibodies (eBiosciences).

Statistical Analysis. Data was analyzed using GraphPad Prism 5 software, represented as mean with standard error, and assessed for significance using the Mann-Whitney *U* or student T test statistics. *P*-values less than .05 were considered statistically significant. *< .05, **< .01, ***< .001

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Conflicts of Interest

Authors declare no financial or commercial conflict of interest.

Figure 2.1. CD3-NK1.1$^+$ cells traffic to the MLN and are activated at sites of infection. C57BL/6 mice were infected orally with 10^8 *C. rodentium* and euthanized at various days post infection. (A) General gating strategy for flow cytometric analysis. (B) The percentage of CD3$^-$NK1.1$^+$ cells in the MLNs harvested from mice at indicated time points after infection, and from uninfected controls was analyzed by flow cytometry. (C) The percentage of CD3$^-$NK1.1$^+$ cells in MLN at day 2 post infection that are IFN-γ^+ are shown. (D) Splenic (top) and lamina propria immune cells of the colon (bottom) are shown. (E, F) Total cell numbers for CD3$^-$NK1.1$^+$IFN-γ^+ cells for the (E) spleen and (F) colon are shown. All cells were gated on CD45$^+$ CD3$^-$ lymphocyte populations and detected for expression of NK1.1 and intracellular IFN-γ as indicated. Data are from one experiment representative of two independent experiments, each performed with n=3 per group. * P<0.05. t-test.

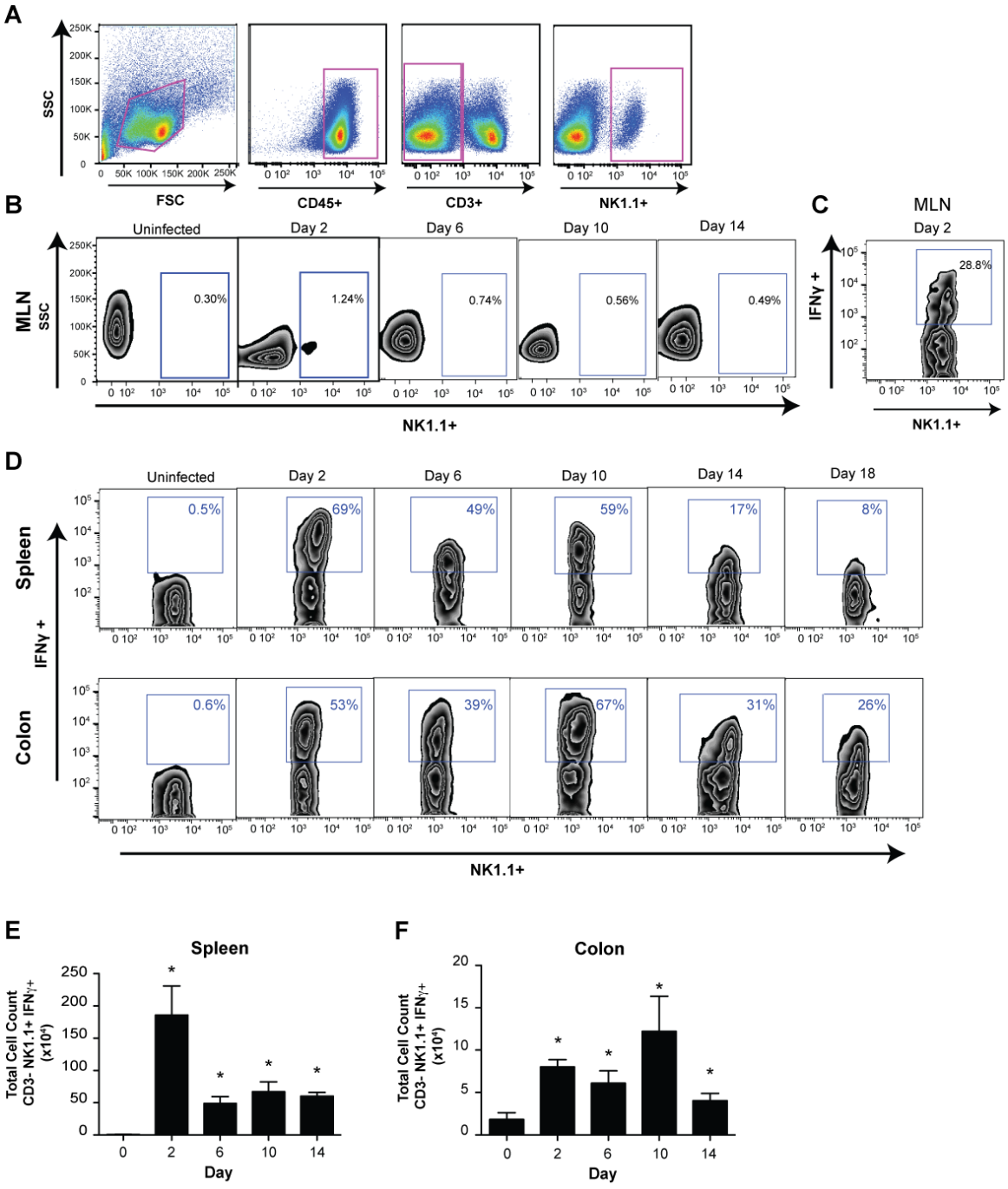

A
SSC
FSC
CD45+
CD3+
NK1.1+
B
MLN
Uninfected
Day 2
Day 6
Day 10
Day 14
0.30%
1.24%
0.74%
0.56%
0.49%
C
MLN
Day 2
28.8%
IFNγ +
D
Uninfected
Day 2
Day 6
Day 10
Day 14
Day 18
Spleen
0.5%
69%
49%
59%
17%
8%
Colon
0.6%
53%
39%
67%
31%
26%
IFNγ +
E
Spleen
Total Cell Count CD3- NK1.1+ IFNγ+ (x10⁴)
Day
F
Colon
Total Cell Count CD3- NK1.1+ IFNγ+ (x10⁴)
Day

Figure 1

Figure 2.2. NK1.1$^+$ cells are important for controlling *C. rodentium* infecton at the peak of the inflammatary response. (A, B) The bacterial load of *Citrobacter rodentrium* in (A) fecal pellets and (B) colon of wild type and NK1.1-depleted mice are shown as mean±SEM of n=5 per group and are from one experiment representative of 2 independent experiments performed. (C) The bacterial load in the spleen of wild type and NK1.1-depleted mice was determined at day 14 after *C. rodentium* infection. Each symbol represents an individual mouse and the bars represent mean±SEM and are from two independent experiments.. (D) Tissue sections from uninfected (left), day 11 p.i. (center), and day 14 p.i. (right) mice were H&E stained. Original magnification 20×. (E) Pathology scores were measured as described in Supporting Information Table 1. SE, surface epithelium; SM, submucosa; M, mucosa; L, lumen; GC, goblet cell. Arrows indicate inflammatory cellular infiltrates (CI), crypt hyperplasia (H), edema (E), and desquamation (D).Data are shown as mean±SEM of 10 mice from two independent experiments. (F) Crypt length was measured as described from infected tissue sections at day 11 and 14 after infection. Data shown are representative of two independent experiments performed. *P<0.05, **P<0.01, ***P<0.001, t-test.

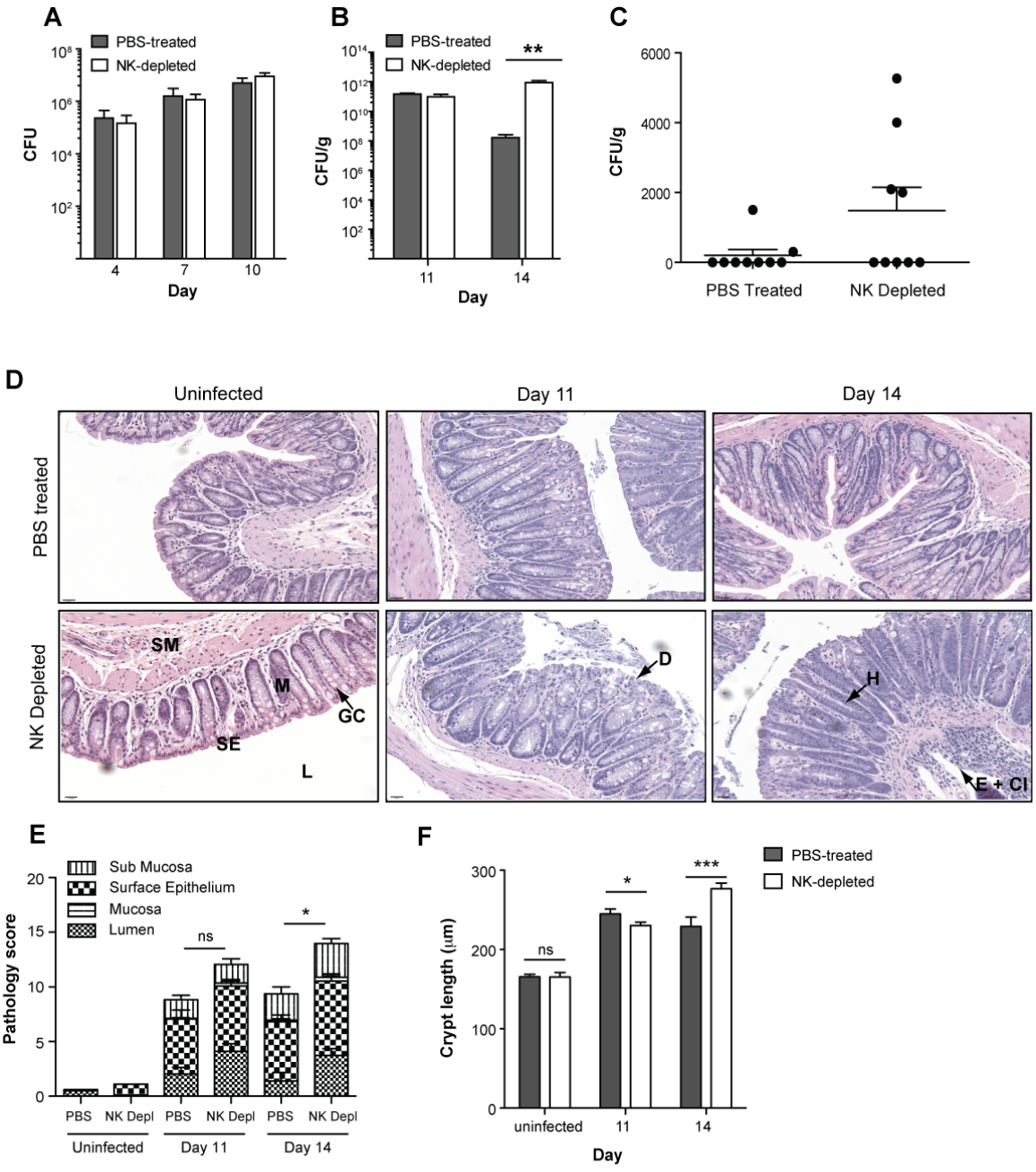

A
PBS-treated
NK-depleted
CFU
Day
B
PBS-treated
NK-depleted
**
CFU/g
Day
C
CFU/g
PBS Treated
NK Depleted
D
Uninfected
Day 11
Day 14
PBS treated
NK Depleted
SM
M
GC
SE
L
D
H
E + CI
E
Sub Mucosa
Surface Epithelium
Mucosa
Lumen
Pathology score
ns
*
PBS
NK Depl
Uninfected
Day 11
Day 14
F
PBS-treated
NK-depleted
Crypt length (μm)
ns
*
***
uninfected
11
14
Day

Figure 2

Figure 2.3. NK1.1$^+$ cells are important for triggering Th1 cytokine and chemokine responses to *C. rodentium* infection. NK1.1-depleted and PBS-treated C57BL/6 mice were infected with *C. rodentium* and euthanized at either day 6 or day 14. Colons were excised, flushed, and incubated for 24 hours in cRPMI at 37°C. Supernatants were analyzed by ELISA for the indicated (A) cytokines (IFN-γ, TNF-α and IL-12p40) and (B) CXCL9/MIG. Data are shown as mean±SEM of 6 samples pooled from 2 independent experiments, each performed with n=3 per group. * $P<0.05$, ** $P<0.01$, t-test.

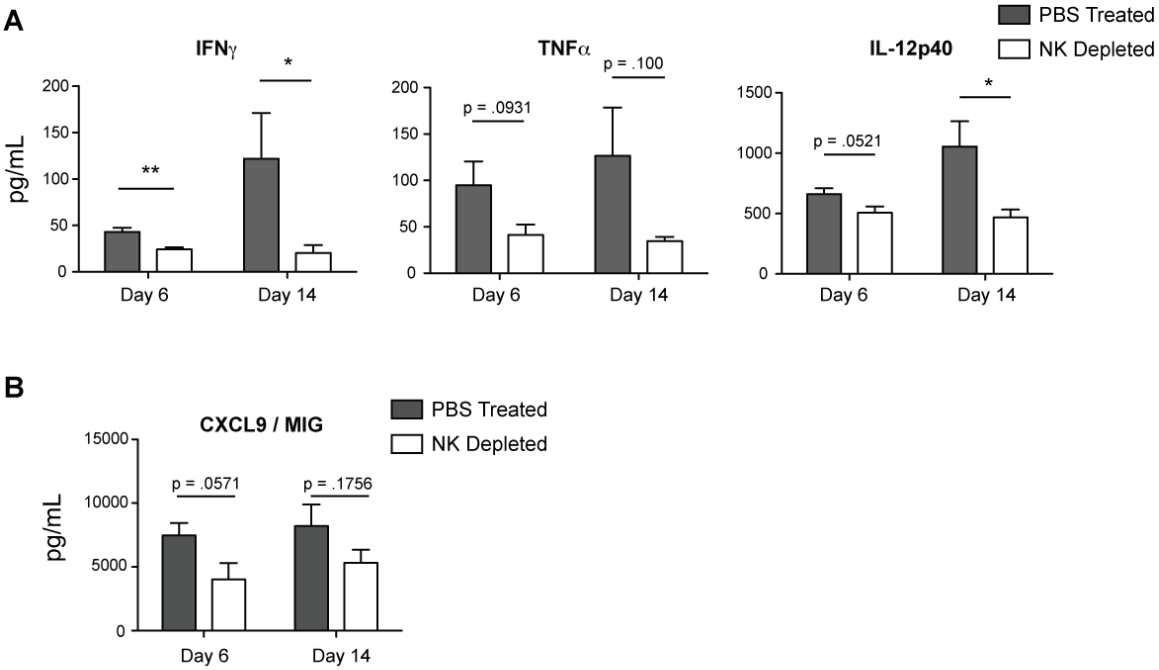

A
IFNγ
TNFα
IL-12p40
PBS Treated
NK Depleted
pg/mL
**
*
p = .0931
p = .100
p = .0521
*
Day 6
Day 14
B
CXCL9 / MIG
PBS Treated
NK Depleted
pg/mL
p = .0571
p = .1756
Day 6
Day 14

Figure 3

Figure 2.4. Lower IgG responses in NK1.1-depleted mice. Plasma was collected from cardiac punctures of NK1.1-depleted and PBS-treated C57BL/6 mice infected with *C. rodentium* and euthanized at indicated time points. Total anti-*Citrobacter* IgG titre was determined by ELISA. Data are shown as mean±SEM of 6 samples pooled from two independent experiments performed, each performed with n=3 per group. ** $P<0.01$, t-test.

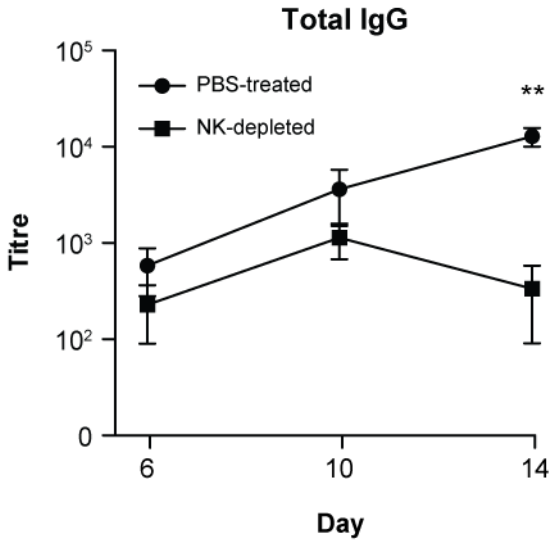

Total IgG
PBS-treated
NK-depleted
**
Titre
10^5
10^4
10^3
10^2
0
6
10
14
Day

Figure 4

Figure 2.5. Loss of NK1.1$^+$ cells results in decreased Th1 CD4+ T cell responses. NK1.1-depleted and PBS-treated C57BL/6 mice were infected with *C. rodentium* and euthanized at various time points. (A) CD4$^+$ T-cell staining in the distal colon in wild type and NK-depleted mice euthanized at day 14 is shown. Original magnification, 20×. (B) The number of CD4$^+$ cells per view was scored using ImageJ software and shown as mean±SEM of 6 samples pooled from two independent experiments, with n=3 per group. (C) The percentage of IFN-γ-expressing CD4$^+$ T cells within the lamina propria (LP) of colons from PBS-treated (top) and NK-depleted (bottom) mice were analyzed by flow cytometry. Cells were gated on CD45$^+$ CD3$^+$ lymphocyte populations and detected for expression of CD4 and intracellular IFN-γ. (D) Total cell numbers from indicated time points from (C) are shown as mean±SEM of 6 samples pooled from two independent experiments, with n=3 per group. *** P<0.001,t-test.

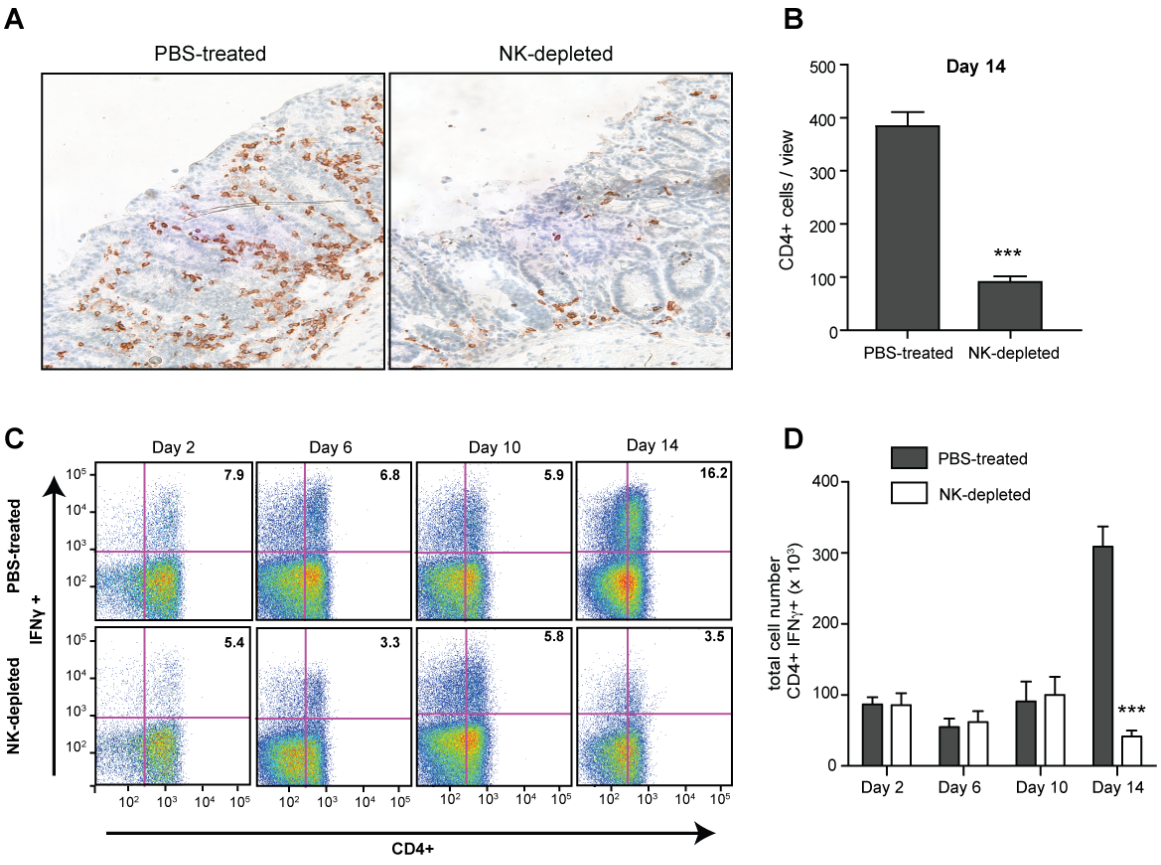

A
PBS-treated
NK-depleted
B
Day 14
CD4+ cells / view
500
400
300
200
100
0
***
PBS-treated
NK-depleted
C
Day 2
Day 6
Day 10
Day 14
PBS-treated
NK-depleted
IFNγ +
7.9
6.8
5.9
16.2
5.4
3.3
5.8
3.5
CD4+
D
PBS-treated
NK-depleted
total cell number
CD4+ IFNγ+ (x 10³)
400
300
200
100
0
***
Day 2
Day 6
Day 10
Day 14

Figure 5

Figure 2.6. Depletion of NK1.1$^+$ cells does not affect long-term clearance due to delayed development of a Th1 response. NK1.1-depleted and PBS-treated C57BL/6 mice were infected with *C. rodentium* and euthanized at day 18, and 21. (A) Bacterial load in the colon was determined at each time point. Data are shown as mean±SEM of n=8 pooled from two independent experiments, each performed with 4 per group. (B) Sections of distal colon from each mouse were taken for histological analysis, H&E stained, and each image (original 10× magnification) is representative of the indicated group: day 18 p.i. (left), day 21 p.i. (right). (C) Pathology scores were determined as described and (D) crypt lengths of 30 well-oriented crypts per mouse were determined. Data are shown as mean±SEM of 8 mice pooled from two experiments. (E) The percentage and (F) total cell number of IFN-γ-expressing CD4$^+$ T cells within the lamina propria (LP) of colons from PBS-treated (top) and NK1.1-depleted (bottom) mice at day 18 after infection as analyzed by flow cytometry. Cells were gated on CD45$^+$ CD3$^+$ lymphocyte populations and detected for expression of CD4 and intracellular IFN-γ. (F) Data are shown as mean±SEM of 6 mice pooled from two independent experiments. * $P<0.05$, t-test.

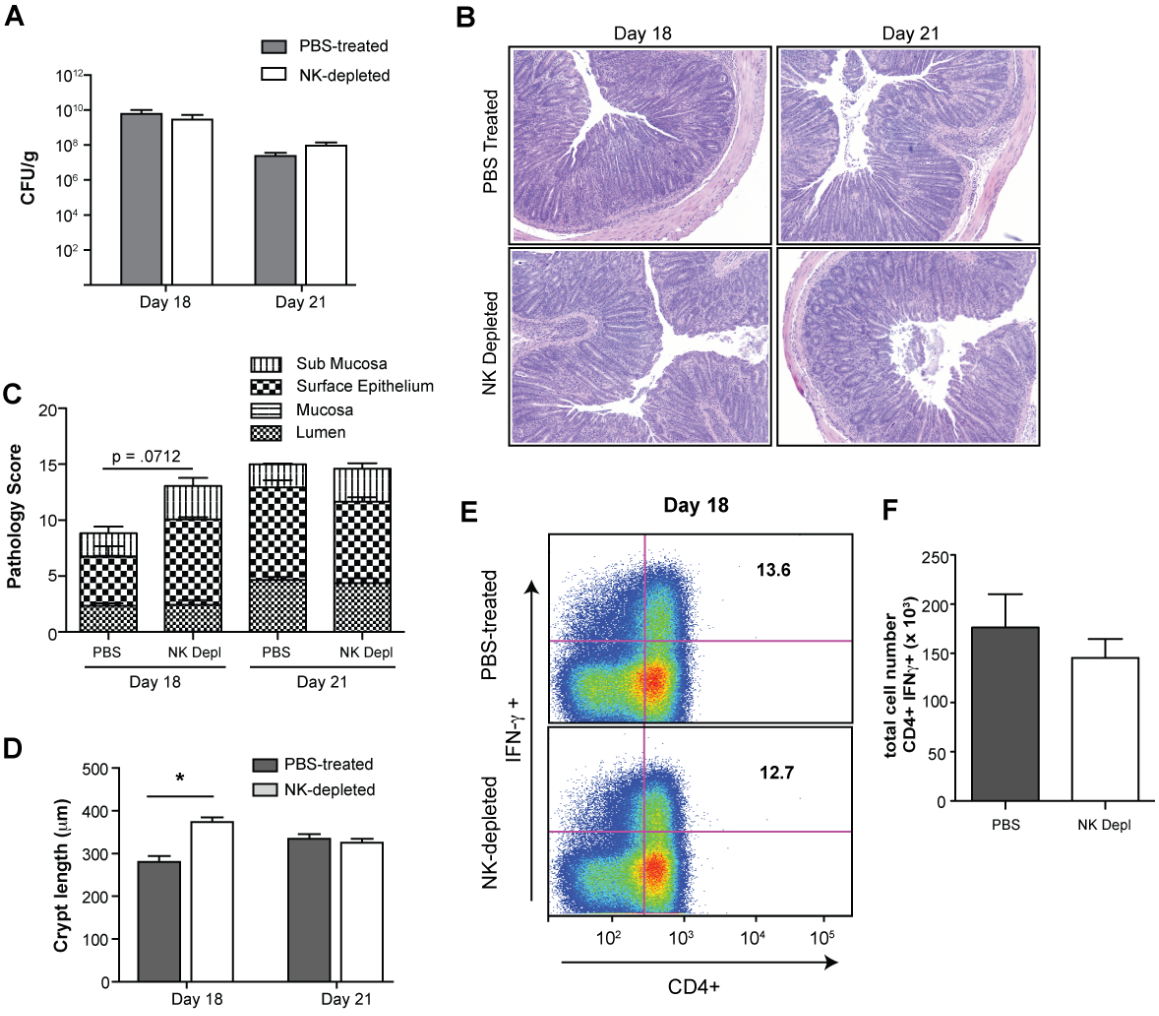

A
PBS-treated
NK-depleted
CFU/g
Day 18
Day 21
B
Day 18
Day 21
PBS Treated
NK Depleted
C
Sub Mucosa
Surface Epithelium
Mucosa
Lumen
Pathology Score
p = .0712
PBS
NK Depl
PBS
NK Depl
Day 18
Day 21
D
PBS-treated
NK-depleted
Crypt length (μm)
*
Day 18
Day 21
E
Day 18
PBS-treated
NK-depleted
IFN-γ +
13.6
12.7
CD4+
F
total cell number CD4+ IFNγ+ (x 10³)
PBS
NK Depl

Figure 6

Figure 2.7. Increased susceptibility of NK1.1-depleted mice is dependent on IFN-γ. NK1.1-depleted and PBS-treated IFN-$\gamma^{-/-}$ mice were infected with *C. rodentium* and euthanized on day 14. (A, B) Bacterial loads in the (A) colon and (B) spleen were determined and shown as mean±SEM of 8 samples pooled from two independent experiments, each performed with n=4.

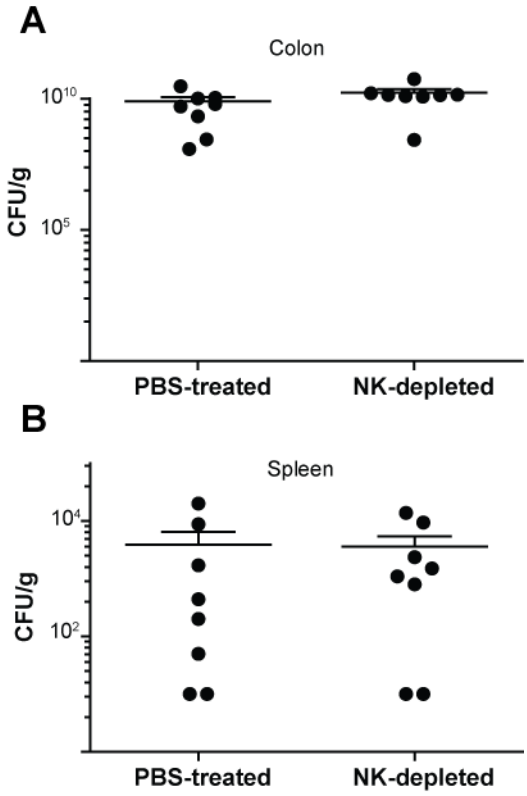
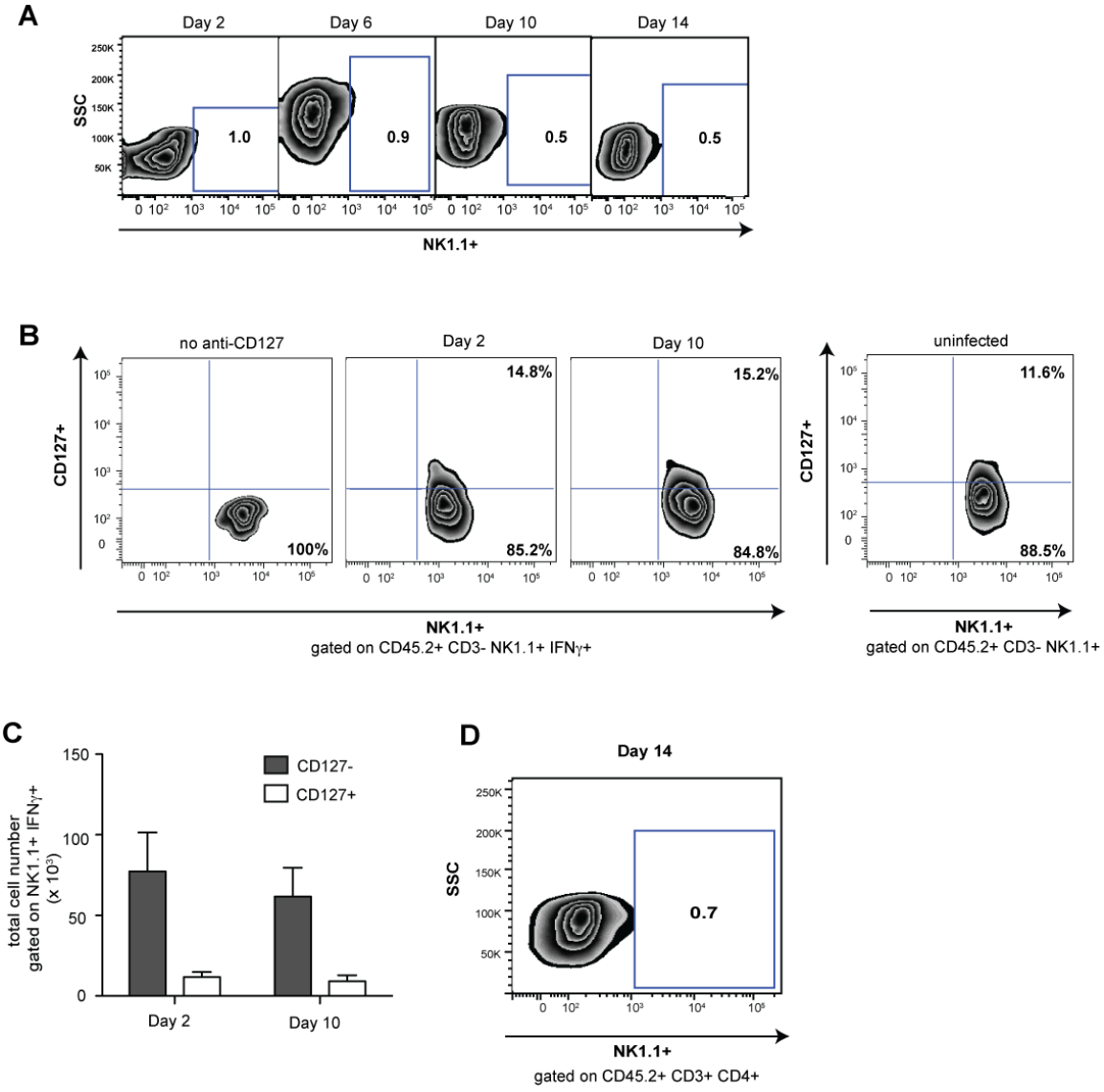

A
Colon
10^{10}
10^{5}
CFU/g
PBS-treated
NK-depleted
B
Spleen
10^{4}
10^{2}
CFU/g
PBS-treated
NK-depleted

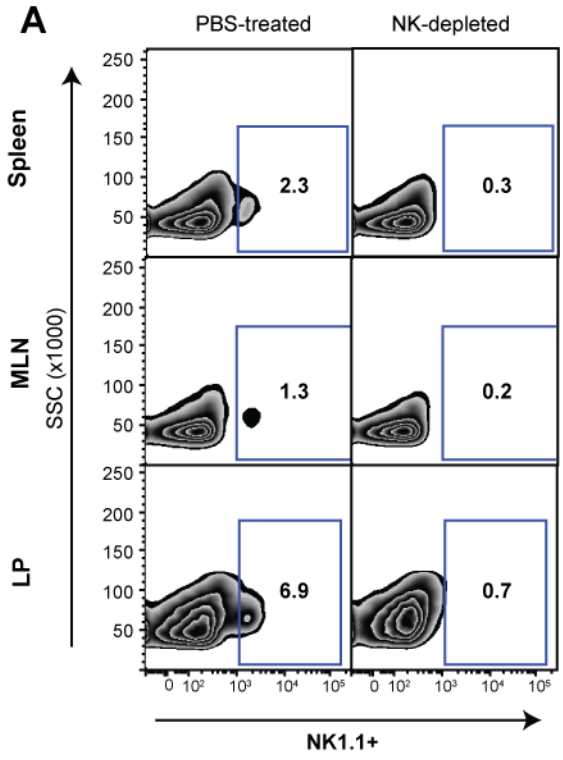
Figure 7

Figure 2.S1. NK1.1+ populations present during infection. C57BL/6 mice were infected with C. rodentium (n=3 per group) and euthanized at various time points. (A) Lymphocytes from the lamina propria of the colons of infected mice were isolated from indicated time points and CD3+NK1.1+ responses were monitored. All cells are gated on CD45+CD3+ cells. (B) Lymphocytes from the lamina propria were isolated at indicated time points, and stained for NK-like ILC1 marker, CD127. All cells were gated on CD45+CD3-NK1.1+IFNγ+ cells, except for uninfected control which was gated on CD45+CD3-NK1.1+ as there was no appreciable IFNγ+ population. No anti-CD127 panel is the fluorescence minus one control for this antibody, and is representative of the isotype control. (C) Total cell numbers representation of data from (B). (D) Lymphocytes from the lamina propria of the colons of infected mice were isolated at day 14 after infection and stained for NK1.1+ CD4+ T cells. All cells gated on CD45+ CD3+ CD4+ lymphocytes. All data are representative of two independent experiments.



Supplemental Figure S1

Figure 2.S2. Confirmation of depletion of NK1.1+ populations in various organs. C57BL/6 mice (n=3 per group) were infected with C. rodentium and euthanized at various time points. (A) Lymphocytes from the spleen, MLN, and lamina propria of the colon were isolated and stained for NK1.1 expression. All cells are gated on CD45+ lymphocytes. Data is representative of all time points examined. All data are representative of two independent experiments.



Supplemental Figure S2

Table 2.S1. Histopathological scoring matrix

Layer	Pathological Characteristics	Maximum Score (in parentheses)
(*i*) Lumen	empty	(0)
	necrotic epithelial cells	scant (1)
		moderate (2)
		dense (3)
	PMNs	scant (2)
		moderate (3)
		dense (4)
(*ii*) Surface Epithelium	no pathological changes	(0)
	regenerative changes	mild (1)
		moderate (2)
		severe (3)
	desquamation	patchy (1)
		diffuse (2)
	PMNs in epithelium	(1)
	Goblet cells (GC) (40x)	0 = >28 goblets cells/field
		1 = 11-28 GC/field
		2 = 1-10 GC/field
		3 = <1 GC/field
	Ulceration	(1)
(*iii*) Mucosa	No pathological changes	(0)
	Crypt abscesses	Rare, >15% (1)
		Moderate, 15-20% (2)
		Abundant, >50% (3)
	Presence of mucinous plugs	(1)
	Presence of granulation tissue	(1)
(*iv*) Submucosa	No pathological changes	(0)
	Mononuclear cell infiltrate	1 small aggregate (0)
		>1 aggregate (1)
		large aggregates plus single cells (2)
	PMN infiltrate	No extravascular PMNs (0)
		Single extravascular PMNs (1)
		PMN aggregates (2)
	Edema	Mild (0)
		Moderate (1)
		Severe (2)

Chapter Three

CXCL9 contributes to antimicrobial protection of the gut during *Citrobacter rodentium* infection

Chapter Three – Co-authorship Statement

Chapter Three consists of the following manuscript:

Reid-Yu, SA, Tuinema, BR, Small, CLN, Xing, L, and BK Coombes. **CXCL9 contributes to antimicrobial protection of the gut during *Citrobacter rodentium* infection**.

At the time of this thesis publication, this manuscript was intended for submission in September 2014 to the journal, *PloS Pathogens*.

The following work was completed by individuals other than myself:

1. *In vitro* bacterial killing assays were performed by B.R.T. and S.A.R-Y.
2. Generation of Δ*phoPQ C. rodentium* mutant was performed by B.R.T.
3. *In vitro* co-culture BM experiments were performed by C.L.N.S. and L.X.

CXCL9 contributes to antimicrobial protection of the gut during *Citrobacter rodentium* infection

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Abstract

Chemokines have been shown to be effective bactericidal molecules against a variety of bacteria and fungi, *in vitro*. These direct antimicrobial effects are independent of their chemotactic activities involving immunological receptors. However, the direct biological role that these proteins may play in host defense, particularly against intestinal pathogens, is poorly understood. Here, we show that CXCL9, an ELR- chemokine, exhibits direct antimicrobial activity against *Citrobacter rodentium*, an attaching/effacing pathogen that infects the gut mucosa. Inhibition of this antimicrobial activity *in vivo* using anti-CXCL9 antibodies increases host susceptibility to *C. rodentium* infection with pronounced bacterial penetration into crypts, increased bacterial load, and worsened tissue pathology. Using $Rag1^{-/-}$ mice, and $CXCR3^{-/-}$ mice, we demonstrate that the role for CXCL9 in protecting the gut mucosa is independent of an adaptive response, or its immunological receptor, CXCR3. Finally, we provide evidence that phagocytes function in tandem with NK cells for robust CXCL9 responses to *C. rodentium*. These findings identify a novel role for the immune cell-derived CXCL9 chemokine in directing a protective antimicrobial response in the intestinal mucosa.

Author Summary

Host defense peptides are an essential part of the innate immune response to pathogens, particularly at mucosal surfaces. Some chemokines, previously known for their ability to recruit immune cells to a site of inflammation, have been identified that have direct antimicrobial activity *in vitro* against a variety of pathogens. Despite this, it was unknown whether chemokines play a role in protecting the gut mucosa, independent of their immunological receptors, against enteric pathogens. We demonstrated a direct antimicrobial role for CXCL9 against the intestinal pathogen, *Citrobacter rodentium*. Neutralization of CXCL9-dependent antimicrobial activity *in vivo* increased host susceptibility to infection, leading to bacterial penetration into intestinal crypts and increased tissue pathology. These data support the importance of a receptor-independent role for chemokines in host defense at mucosal surfaces.

Introduction

The intestinal tract is a site of continuous interaction between host and microbe. Tight regulation of immune surveillance and activation is essential for maintaining the integrity of this interface during non-infectious periods while preserving the ability to launch immediate action upon exposure to a pathogen in order to protect the host from damage. Chemokines are a vital component of this protective response. These chemoattractants link innate and adaptive arms of the host immune system by activating and recruiting immune cells to sites of infection [95]. Until recently, the activity of these molecules focused solely on their chemotactic properties, which they orchestrate upon interaction with their cognate receptors on various immune cells [95]. However, mounting evidence has shown a direct antimicrobial function for a number of chemokines that relates to their cationic surface properties, similar to antimicrobial host defense peptides [99,101].

Host defense peptides (or antimicrobial peptides), are produced by a wide variety of cell types and form an important component of innate immune defenses in many forms of life [273,274]. Although the exact bactericidal mechanism for cationic antimicrobial peptides remains debated [83], membrane-disrupting activity appears to be a common feature, facilitated by cationic charge distribution and amphipathicity, allowing for attachment to and insertion into bacterial membranes. In mammals, antimicrobial peptides are particularly important in protection of the gut mucosa against infection, and in maintenance of intestinal homeostasis [275]. Of the chemokines reported to exhibit

bactericidal activity, CXCL9 appears to be one of the more potent members, with a cationic C-terminal domain (+18 charge over 35 residues) that resembles other known cationic antimicrobial peptides [101,276]. *In vitro*, CXCL9 has the greatest bactericidal activity against *Escherichia coli*, *Listeria monocytogenes*, and *Bacillus anthracis* [101,105]. Additionally, a potential antimicrobial function for CXCL9 has been examined in respiratory, wound, and urogenital pathogens, where it appears to play a key role in protection of mucosal surfaces against pathogen infection [105,276-278]. However, outside of its role in T cell homing and activation, a potential role for CXCL9 in conferring antimicrobial protection of the gut mucosae against intestinal infections has not been investigated.

Citrobacter rodentium is a murine intestinal pathogen used extensively to model infections with the attaching and effacing (A/E) human pathogens, enteropathogenic *E. coli* and enterohemorrhagic *E. coli* [279]. *C. rodentium* colonizes the cecum prior to traversing to the primary site of infection in the distal colon [30]. Like other A/E pathogens, *Citrobacter* forms intimate attachments with the epithelial surface by using a type III secretion system and effectors that it injects into the host's intestinal epithelial cells [280]. The host response to *C. rodentium* infection includes an early and robust chemokine response [281-283]. Elimination of infection in resistant murine hosts is dependent upon Th1 adaptive responses [136,190,280] mediated by the IFNγ-stimulated chemokines CXCL9, CXCL10, and CXCL11 acting in a CXCR3 dependent manner [284]. Previous work by our laboratory found that loss of IFNγ-producing NK1.1+ CD3-

cells, and decreased CXCL9 levels, correlated with delayed Th1 responses, reduced CD4+ T cell homing to the colonic tissue, and host susceptibility to infection [190]. Similarly, treatment of p38$\alpha^{\Delta T}$ mice, which have decreased IFNγ responses to *Citrobacter* infection, with exogenous IFNγ, increases ELR- chemokine production and T cell homing to site of infection [285]. Interestingly, of all the ELR- chemokines, CXCL9 is the most highly expressed during *C. rodentium* infection [281], suggesting it plays an important role in protecting the host from this intestinal pathogen. Whether this is solely related to its characteristic chemotactic activity, or additional unstudied aspects of antimicrobial innate immunity, is not known. Here, we study a direct antimicrobial function of CXCL9, independent of its receptor, which protects the gut mucosae from infection, and limits bacterial penetration into crypts. Our results identify a novel function for CXCL9 in innate antimicrobial defense of the intestinal mucosa.

Results

CXCL9 directs *C. rodentium* killing *in vitro*.

ELR- chemokines have an emerging role as potent antimicrobial agents, owing to their cationic C-terminal domain rich in positively charged amino acids. Since the level of CXCL9 increases dramatically following *Citrobacter* infection of resistant mice [281], we first determined whether CXCL9 exerted antimicrobial activity against *C. rodentium in vitro*. Treatment of *C. rodentium* with CXCL9 resulted in a dose-dependent bacterial killing, as measured by viable colony counts of residual survivors (**Figure 1A**). Exposure to CXCL9 at ~4 μg/ml (270 nM) resulted in 100% killing, and 85% killing at ~0.4 μg/ml (27 nM). This concentration is biologically relevant as CXCL9 levels in rectal perfusions from the inflamed human intestine can reach up to 2 μg/ml (138 nM) [286]. Time-kill curves showed that killing was rapid, reaching near maximal effect at ~5 min post-exposure to 27 nM CXCL9 (**Figure 1B**). Many host-adapted pathogens have evolved resistance to antimicrobial peptides through LPS modifications controlled by the two-component system PhoP-PhoQ [287-289]. To determine whether residual survival of *C. rodentium* following treatment with lower doses of CXCL9 was PhoP-PhoQ dependent, we treated wild type bacteria and a *phoPQ* deletion mutant with a concentration of CXCL9 that produced ~10-20% survival of wild type bacteria, and measured viable bacteria after 1-2 h. The Δ*phoPQ* strain was more susceptible to CXCL9, with only 1-2% residual survival, similar to a peptide-sensitive strain of *E. coli*. A similar result was seen using the control human α-defensin peptide (**Figure 1C**). Interestingly, we found that a 1000-fold lower molar concentration of CXCL9 could elicit similar killing when

compared with α-defensin. To confirm that this killing was directly linked to CXCL9, we performed killing assays in the presence of purified anti-CXCL9 antibody or control IgG. Whereas CXCL9 killed ~100% of *C. rodentium,* this activity was completely blocked by anti-CXCL9 antibody but not an IgG control (**Figure 1D**). Antimicrobial peptides like polymyxin B kill bacteria by inducing membrane permeability [290], which can be measured by a fluorescence increase following membrane incorporation of the neutral hydrophobic molecule 1-anilino-8-naphthalene-sulfonate (ANS) [291]. Indeed, injection of polymyxin B into a culture of *C. rodentium* led to an immediate increase in ANS fluorescence (**Figure 1E**). To better understand how CXCL9 might exert its antimicrobial activity, we measured ANS fluorescence following CXCL9 injection into *C. rodentium* culture. An increase in fluorescence, which occurs when ANS partitions into exposed membrane, was observed on a similar time scale and magnitude upon injection of CXCL9 as that seen with polymyxin B (**Figure 1E**). Together, these data established a direct antimicrobial activity for CXCL9 on *C. rodentium* in a manner consistent with membrane disruption, similar to classic antimicrobial peptides.

CXCL9 increases host survival and decreases *C. rodentium* burden *in vivo.*

Previous work investigating the role of CXCL9 in the gut has focused solely upon its chemotactic properties. However our *in vitro* results suggested that CXCL9 might have biological significance as a direct antimicrobial molecule. To investigate this, we depleted CXCL9 in $Rag1^{-/-}$ mice infected with *C. rodentium* and monitored host survival and bacterial load. $Rag1^{-/-}$ mice were used in order to study the protective effect of

CXCL9 independent of its ability to recruit T cells. *C. rodentium*-infected mice depleted of CXCL9 died, on average, 2 days earlier than mice receiving IgG control. This was significant, and specific to CXCL9 depletion, as depletion of another ELR- chemokine, CXCL10, had no effect on host mortality over 10 days (**Figure 2A**). In line with these data, *C. rodentium* burden was 10-100 times higher in the fecal output from CXCL9-depleted mice compared to controls over the 10-day infection period (**Figure 2B**). In order to confirm that anti-CXCL9 antibodies were reaching the lumen of the gut, we measured IgG levels in fecal samples following intraperitoneal delivery of anti-CXCL9 antibody or control IgG into uninfected $Rag1^{-/-}$ mice and showed an IgG accumulation in the feces (**Figure S1**). As a secondary biological readout for CXCL9 depletion, we measured the number of infiltrating CD3+ cells in the distal colon 10 days after *C. rodentium* infection of immunocompetent C57BL/6 mice and in mice depleted of CXCL9. As expected, in *C. rodentium*-infected immunocompetent C57BL/6 mice depleted of CXCL9, there was a ~75% reduction in the number of CD3+ cells in the distal colon (**Figure S1**).

To investigate the impact of CXCL9 depletion on disease severity, we examined the gross pathology of the gut during necropsy. *C. rodentium*-infected mice depleted of CXCL9 had increased evidence of colitis in the cecum, which was shrunken and partially emptied; shortening and thickening of the colon; increased incidence of watery stool; and hematomas along the length of the cecum and colon (**Figure 2C**). As a measure of diarrhea in the stool, we measured fecal water content at day 10, which was significantly greater in CXCL9-depleted mice compared to controls (**Figure 2D**). These results were

independent of significant differences in the levels of TNFα, IFNγ, IL12p40, or IL-10 in colonic explants on day 10 post-infection, which were all similar in control mice and mice depleted of CXCL9 (**Figure S2**). Together, these data established an important role for CXCL9 in host defense against *C. rodentium* infection and in limiting *C. rodentium* burden in the gut.

CXCL9 protects the gut from *C. rodentium*-induced pathology and bacterial penetration into crypts.

The pathological differences in *C. rodentium*-infected mice upon CXCL9 depletion suggested a worsened immunopathological response to infection. Since the pathologic impact on the host can be affected by less than a log-change in peak *C. rodentium* load [292], we scored colonic histopathology on day 10 in *C. rodentium*-infected control mice and animals depleted of CXCL9. CXCL9 depletion was associated with increased numbers of necrotic epithelial cells in the lumen and immune cell infiltration, with destruction of the epithelial architecture (**Figure 3A**). These changes resulted in significantly greater transmural pathology in the colon (**Figure 3B**). Similar findings were observed for cecal pathology upon *C. rodentium* infection of CXCL9-depleted mice, with a more pronounced pathology in the mucosa and submucosa regions of depleted animals (**Figure S3**). In a typical *C. rodentium* infection, the bacteria attach to the intestinal epithelium, but do not commonly penetrate deep into intestinal crypts [235,293]. To localize *C. rodentium* in infected mice in the presence or absence of CXCL9, we performed immunohistochemical localization of *C. rodentium* using an

antibody specific to *C. rodentium* LPS. Indeed, we observed the majority of *C. rodentium* in close association with the colonic epithelial cell surface in uncontrived Rag1$^{-/-}$ mice, with only marginal evidence of crypt penetration. In contrast, in mice depleted of CXCL9, *C. rodentium* was commonly found to penetrate deeply into crypts in the colon (**Figure 3C and 3D**) and cecum (**Figure S3**).

CXCL9 is mainly dependent on IFNγ for its expression [294], and IFNγ$^{-/-}$ mice have impaired resistance and greater pathology following *C. rodentium* infection similar to that seen in our CXCL9 depletion studies [254]. Given our results following infection of CXCL9-depleted mice, we hypothesized that IFNγ$^{-/-}$ mice would be similarly susceptible to crypt penetration by *C. rodentium* due to the attendant decrease in CXCL9 expression. We tested this by localizing *C. rodentium* in colonic tissues of IFNγ$^{-/-}$ mice and C57BL/6 wild type controls by immunohistochemical staining. In these experiments, we found that *C. rodentium* was localized mainly to the epithelial surface in C57BL/6 mice, whereas bacteria were commonly found penetrating into colonic crypts of IFNγ$^{-/-}$ mice (**Figure 3E and 3F**). Together, these data indicated that the antimicrobial action of CXCL9 helps maintain epithelial barrier defenses against *C. rodentium* by preventing crypt penetration by invading bacteria. The loss of this defense upon CXCL9 depletion allows for bacterial penetration deep into intestinal crypts with an attendant increase in pathology.

The antibacterial defense of CXCL9 is independent of the CXCR3 chemokine receptor.

To further determine the biological significance of direct antimicrobial activity of CXCL9 during *C. rodentium* infection, we infected CXCR3$^{-/-}$ mice that lack the CXCL9 chemokine receptor and thus do not mount CXCR3-dependent effects following ligand interactions. Based on our prior data, we hypothesized that the host susceptibility to *C. rodentium* infection following CXCL9 depletion would persist in CXCR3$^{-/-}$ mice. Indeed, CXCR3$^{-/-}$ mice depleted of CXCL9 carried a significantly increased burden of tissue-associated *C. rodentium* in the colon (**Figure 4A**). Furthermore, CXCL9-depleted CXCR3$^{-/-}$ mice had increased pathology scores in both the distal colon (**Figure 4B** and **Figure 4C**) and in the cecum (**Figure S4**). In agreement with our previous data for a direct role for CXCL9-mediated protection of intestinal crypts, CXCR3$^{-/-}$ mice were able to restrict *C. rodentium* to the epithelial cell surface with virtually no penetration by bacteria into intestinal crypts. In contrast, depletion of CXCL9 in *C. rodentium*-infected CXCR3$^{-/-}$ mice produced a striking invasion of bacteria deep into intestinal crypts in both the colon (**Figure 4D and 4E**) and the cecum (**Figure S4**). These data confirmed that the antibacterial activity and host protection afforded by CXCL9 was independent of CXCR3-ligand-mediated effects.

IFNγ produced by NK cells and macrophages is necessary to achieve maximal CXCL9 expression in response to *C. rodentium*.

Previous work examining transcript levels of CXCL9 in the *C. rodentium* infected colon found the predominant source to be CD11c+ cells, and was therefore attributed dendritic cells (DCs) [281]. However, recent work into understanding the role that DCs and macrophages play in intestinal homeostasis, as well as inflammation, has revealed that the intestinal tract is more heavily populated with macrophages [220], and that some previous work attributing function to CD11c+ DCs has instead been misidentified macrophages [295-297]. Indeed, macrophages have been shown to be a significant source of CXCL9 in other inflamed tissues [298,299]. We measured CXCL9 release from bone marrow-derived DC (BMDC) and macrophages (BMDM) in response to heat-killed *Citrobacter* and IFNγ. Unstimulated BMDC and BMDM did not produce detectable levels of secreted CXCL9. Exposure to either IFNγ alone, or *C. rodentium* alone stimulated intermediate levels of CXCL9, which was significantly boosted in response to both stimuli in combination (**Figure 5A**). These data were consistent with that of others, showing that maximal CXCL9 expression is induced by IFNγ, in combination with additional microbial stimuli [300,301].

We next examined the potential sources of IFNγ responsible for driving CXCL9 expression within DCs, and macrophages. It is well established that natural killer (NK) cells are an important early source of IFNγ, and previous work by our laboratory has shown that depletion of NK1.1+ cells, a common NK cell marker, in *Citrobacter*-infected mice results in decreased CXCL9 expression in the colon [190]. Unstimulated BMDM, BMDC, or NK cells did not express CXCL9. However, co-culture of BMDM and NK cells, or DCs and NK cells, lead to CXCL9 secretion that was dependent on the presence

of *C. rodentium* (**Figure 5B**). NK cells were important for maximal CXCL9 release as CXCL9 levels were reduced by ~50% in the absence of NK cells. Interestingly, we observed that macrophages were far more capable at not only inducing their own expression of CXCL9 upon exposure to *C. rodentium*, independent of NK cells, but also released more CXCL9 upon co-stimulation with either IFNγ and *Citrobacter*, or NK cells and *Citrobacter* in paired experiments directly comparing macrophages and DCs (**Figure 5B**).

Previous studies have shown that macrophages are capable of producing IFNγ in a TLR signaling-dependent fashion [204,302]. Using NK cells isolated from wild type mice, or IFNγ$^{-/-}$ animals and BMDM derived from these mice, we measured the contribution IFNγ produced by macrophages and NK cells on CXCL9 release following *C. rodentium* stimulation. We found that IFNγ expression by both NK cells and macrophages was critical for maximum CXCL9 release in the presence of heat-killed *C. rodentium* (**Figure 5C and 5D**). Equivalent CXCL9 levels were released from *C. rodentium*-stimulated BMDM from wild type mice when NK cells were absent, or in the presence of IFNγ-deficient NK cells. These data indicated that the release of CXCL9 was dependent upon IFNγ expression by NK cells, and not other co-stimulatory, and/or NK cell-directed alternative cytokine expression mechanisms. Macrophage-derived IFNγ was also important for this response as release of CXCL9 was significantly reduced by ~50-60% from macrophages unable to produce their own IFNγ (**Figure 5D**). This was further supported in experiments that showed CXCL9 release from *C. rodentium*-stimulated

IFNγ$^{-/-}$ macrophages was significantly less than CXCL9 levels from wild type macrophages (**Figure 5C**). Together, these data indicated that IFNγ produced by both NK cells and macrophages is necessary to achieve maximal CXCL9 expression in response to *C. rodentium*. Finally, we measured CXCL9 levels in the gut following *C. rodentium* infection and found that, similar to our *in vitro* results, chemokine levels in the cecum and colon were significantly blunted in IFNγ$^{-/-}$ mice (**Figure 5E**). Together, these data indicate that IFNγ produced by NK cells and macrophages is necessary to achieve maximal CXCL9 expression in response to *C. rodentium.*

Discussion

The intestinal tract is an environment that requires a balanced set of immunological responses, capable of tolerating the host's commensal microbiota, while remaining primed to respond to invasion by pathogenic bacteria. Early immune responses to pathogens are critical for controlling both bacterial burden, and disease pathology, which the host achieves through combined cellular and innate antimicrobial responses. Chemokines are an important facet to this host protection, by linking innate antimicrobial activity with cellular homing to the site of infection. We found that CXCL9, a chemokine known previously as an important modulator of CXCR3-dependent cellular homing following *C. rodentium* infections, has an important additional function in innate antimicrobial defense of the gut. This antimicrobial activity is independent of the CXCR3 receptor, or other aspects of adaptive immunity, and helps to control bacterial burden while protecting intestinal crypts from pathogen invasion.

IFNγ$^{-/-}$ mice are more susceptible to *C. rodentium* infection [254]. The basis for this was thought to be the loss an IFNγ-dependent antimicrobial factor expressed in colon. Our results are consistent with CXCL9, a chemokine induced by IFNγ in combination with other microbial stimuli, as a likely mediator of host protection in IFNγ-competent hosts. Previous worked showed that p38α expression in T cells regulates host defense against *C. rodentium* infection [285]. This study revealed that T cells lacking p38α had a significant reduction in IFNγ production following *C. rodentium* infection, a decreased infiltration of inflammatory cells into the colon, and yet increased tissue damage, a result that could be linked to the increased invasion of *C. rodentium* in mice with p38α T cell

deficiency. Interestingly, treatment of these mice with IFNγ restored host defenses against *C. rodentium,* leading to lessened tissue damage, and more importantly, normalization of the tissue-associated bacterial burden. While the authors attributed these findings to increased T cell homing to site of infection, an alternative or additional interpretation, given our current results, is the attendant increase in innate defense mediated by IFNγ-stimulated CXCL9 release.

The increased tissue damage resulting from loss of IFNγ, despite the blunted immune cell infiltration [190,285], is likely due to the pathogen itself gaining access to the privileged host niche within intestinal crypts. In a typical *C. rodentium* infection of resistant hosts, the host restricts the pathogen to the lumen or epithelial surface. However, depletion of CXCL9, or loss of IFNγ production allows for *C. rodentium* to penetrate deep within the crypts. A correlation between invasion of *C. rodentium* into crypts and increased host pathology has been observed in other studies [303,304] and so protecting this niche against intestinal pathogens is a key function for the innate immune system.

Of note, we found that IFNγ$^{-/-}$ mice were capable of producing a modest, but non-protective level of CXCL9 in the colon following *C. rodentium* infection. Although CXCL9 production is typically considered to be dependent on IFNγ, an alternative induction pathway in macrophages involving IFNα/β signaling has been described. For example, low-level expression of CXCL9 was described in IFNγ$^{-/-}$ mice following infection with vaccinia virus [305]. In addition, STAT1 activation by IFNα in primed macrophages also boosts CXCL9 expression [306]. The quantitative contribution of this

IFNγ-independent production of CXCL9 on host protection, however, has not yet been defined.

In this work, we observed that macrophages exhibited greater capacity for CXCL9 expression in the presence of microbial stimuli, and/or IFNγ, compared to DCs. Previous observations attributed the greatest levels of CXCL9 transcript to CD11c+ DCs in the *Citrobacter*-infected colon [281]. However this study relied upon CD11b and CD11c markers to differentiate phagocytes, typical surface markers routinely used to identify DCs and macrophage populations. However, recently it has become clear that many markers previous attributed to a homogenous phagocyte populations, in particular CD11c and CD11b, are expressed on multiple cell types [295]. Therefore, further examination, and in particular direct cell staining for CXCL9 expression, is necessary to identify the population(s) essential for robust expression of this chemokine. In addition to DCs, various cell types have been found to express CXCL9, including epithelial cells, neutrophils, and macrophages [298-300,307]. Recently, staining for CXCL9-producing cells within the inflamed tonsils also revealed macrophages to be the predominate source of the chemokine [299]. Preliminary data from our laboratory has shown co-localization of CXCL9 expression with the F4/80 macrophage marker within the colon of *C. rodentium* infected mice (data not shown). Given these data, macrophages appear to be a significant source of CXCL9 within the inflamed colon of *C. rodentium* infected mice, however the utility of current molecular tools to investigate the cellular sources of CXCL9 in the gut appear, in our hands, to be limited. This could potentially be overcome

by directly labeling native CXCL9 in transgenic mice [308], however additional work is required.

Some host-adapted bacteria have evolved mechanisms of resistance towards antimicrobial host defense peptides through enzymatic cleavage-based mechanisms [309,310]. Evidence for bacterial resistance to the antibacterial activity of CXCL9 has also been observed, further implicating it as an innate host defense that can be a selectable target of resistance. For instance, the streptococcal inhibitor of complement (SIC) protein, secreted by *Streptococcus pyogenes,* can inhibit the antimicrobial activity of the CXCL9 C-terminal domain [276]. SufA from *Finegoldia magna* can also block the antimicrobial activity of CXCL9 by cleavage, while leaving its chemotactic activity intact [277]. *C. rodentium* does not appear to have such intrinsic resistance mechanisms; however, it is possible that other host-derived mechanisms may play a role in certain infections. For example, interaction of *Streptococcus dysgalactiae* with human serum albumin blocks some CXCL9-directed killing activity [311]. Whether such a mechanism is relevant in intestinal infections is not known.

In summary, our data indicate that CXCL9 plays an important role in antimicrobial defense in the infected and inflamed gut. This activity, independent of the chemokine receptor CXCR3 or an adaptive immune response, protects the gut from crypt invasion by *C. rodentium* and the tissue damage that ensues. These data add to the growing body of evidence to support this chemokine as an innate antimicrobial defense molecule at mucosal surfaces.

Materials and Methods

Ethics Statement

All animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University.

Animal Infections

Six to eight-week old C57BL/6, Rag1$^{-/-}$, IFNγ$^{-/-}$, and CXCR3$^{-/-}$ mice were purchased from Jackson Laboratories. Survival experiments were performed with 4-week old Rag1$^{-/-}$ mice. All animals were housed in a specific pathogen-free unit under Level 2 conditions at the Central Animal Facility at McMaster University. For all infections, mice received 2 x 10^8 CFU/mL via orogastric gavage from an overnight culture of *Citrobacter rodentium* (DBS100). For infections, bacteria were pelleted, washed, and resuspended in 10 mM HEPES (pH 8.0), 0.9% NaCl. Bacterial burden was monitored at designated time points by fecal output throughout experiments. At day 10 post infection, mice were sacrificed, and *C. rodentium* burden was determined in the cecum and colon as previously described [190]. For antibody neutralization experiments, mice were given either 200 μg/mL rabbit anti-mouse CXCL9, or 200 μg/mL control rabbit IgG on day -1, 0, 1, and then every 3 days via intraperitoneal injection. All neutralization antibodies were column-purified from rabbit antisera, which was a kind gift from Dr. Cory Hogaboam (University of Michigan/Cedars-Sinai Medical Center).

In vitro killing assay

Bacterial killing assays were performed with wild type *C. rodentium*, a Δ*phoPQ* mutant, and *E. coli* K12. The *phoPQ* deletion was generated by Lambda Red mutagenesis according to published methods [312] using primers BRT151 (tta gcc gtc ctt ctg ccc cgg ctg ctg tcg gcc aaa aat gac ctc cat gtg tag gct gga gct gct tcg) and BRT152 (atg cgc gtt ctg gtt gtt gag gat aat gcg tta cta cgt cac cac ctg cat atg aat atc ctc ctt a). Stationary phase (16-18h) cultures were sub-cultured 1:50 in LB, and grown at 37°C with shaking until OD_{600} = 0.5. Bacteria were pelleted, washed and resuspended in 10 mM HEPES buffer (pH 7.4) to a concentration of 10^5 CFU/mL. Killing was initiated by mixing bacteria with 50 μg/mL human α-defensin (HD5; Prospec), CXCL9 (0.39 μg/mL, or otherwise indicated concentration; Peprotech), or sterile water. Bacteria were incubated at room temperature for 2 h, unless otherwise indicated. Cultures were diluted 1:10 with PBS to quench killing, and viable bacterial counts were assessed on solid agar. All data was normalized to the water control and expressed as survival relative to time zero. For CXCL9 neutralization in the bacterial killing assays, 200 μg/mL antibody (or similarly diluted PBS) was pre-incubated with 5 μg/mL CXCL9 for 20 minutes prior to the assay.

ANS Membrane Permeability Assay

To assess integrity of bacterial cellular membranes, the fluorescent probe, 8-anilino-1-naphthylenesulfonic acid (ANS; Sigma-Aldrich) was used according to previous published protocols [313]. In brief, stationary phase cultures of *C. rodentium* were sub-cultured 1:50 in LB, and grown at 37°C with shaking until OD_{600} = 0.5. Bacteria were

pelleted, washed, and resuspended in sterile 10 mM HEPES Buffer (pH 7.4), 5 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma Aldrich), and 5 mM glucose. Bacteria were incubated for 30 min at room temperature. For each sample, 93 μL of bacteria was added to each well of black, clear bottom, 96-well plate (Costar, Corning, Inc.) with 2 μL 3 mM ANS, and fluorescence was monitored on a Synergy HT microplate reader (BioTek) (excitation, 375nm; emission, 510nm). After 5 minutes, 10 μg/mL CXCL9, 10 μg/mL Polymyxin B (Sigma-Aldrich), or water control was injected and fluorescence was monitored for an additional 30 min.

Histochemical analysis

At 10 days post infection, segments of cecal tip or distal colon were collected and either fixed in buffered 10% formalin, or flash frozen in optimal cutting template compound (OCT; Sakura, Fisher). Segments were fixed for 72 h, paraffin-embedded, sectioned into 6 μm slices, and stained with hematoxylin and eosin (H&E), anti-CD3 antibody (1:1000; Labvision), or anti-*Citrobacter* antibody (1:4000; Statens Serum Institute). H&E sections were used for assessing pathology according to published scoring protocols [314]. All fixed sections were visualized using a Leica microscope. A minimum of 6 views were analyzed for each sample. Evaluation of *C. rodentium* crypt invasion was determined through enumeration of bacterially penetrated crypts from 4-6 views per sample. Anti-CD3 treated sections were enumerated using ImageJ software.

Cytokine and IgG Quantification

At day 10 post infection, cecum and colon were removed, flushed of contents, and washed in ice-cold PBS, pH 7.4. Tissues were cut into 5 mm pieces, and placed in 1 mL RPMI, 50 μg/mL gentamicin. Tissues were incubated for 24 h at 37°C, 5% CO_2 . Levels of CXCL9, IFNγ, TNFα, IL-12p40, and IL-10 were assessed using Duoset Quantikine murine ELISA kits (R&D Systems) according to manufacturer's protocols. For IgG analysis of fecal pellet homogenate supernatants, fresh fecal pellets were collected on day 2 post final injection from naïve, uninfected animals receiving two injections of 200 μg/mL anti-CXCL9 antibody, control IgG, or PBS. Fecal pellets were homogenized as described above, and then centrifuged at 14,000 *g* for 20 min. Supernatants were collected, and frozen at -80°C until analysis. IgG ELISA was performed on samples by coating high-binding 96-well plates overnight at room temperature with goat anti-rabbit anti-IgG antibody (1:10,000; MP Bio). Plates were washed 3 times with PBS (pH 7.4), 0.05% Tween-20, and then blocked with PBS, 1% BSA for 1 h at room temperature. Plates were washed, and samples were added and incubated for 2 h at room temperature. For each sample, a 1:2 dilution (in PBS, 1% BSA) was made, and 100 μL was added to each well. After incubation, plate was again washed three times. Bound antibody was detected by addition of anti-rabbit IgG antibody conjugated to HRP (1:10,000; GE Healthcare), and incubated at room temperature for 2 h. Plates were washed, and developed with 100 μL solution A and B (R&D Systems) for 20 min. The reaction was stopped by addition of 50 μL 1N H_2SO_4. Plates were read at 450 nm using a plate reader.

In vitro co-culture of bone marrow-derived cells and NK cells

NK cells were purified from whole splenocytes from wild type C57BL/6 or IFNγ$^{-/-}$ mice using a NK cell (CD49b+) negative selection enrichment kit from StemCell technologies according to manufacturing protocol. Purified NK cells ($1x10^5$) were cultured in the presence or absence of $3x10^5$ bone marrow-derived macrophages (BMDM) or dendritic cells (BMDC) derived from uninfected wild type C57BL/6 mice or IFNγ$^{-/-}$ mice with or without 1 ng/mL IFNγ, heat killed (hk) *C. rodentium* ($3x10^6$ bacteria) and 8 ng/ml recombinant IL2 for 24 h at 37°C in 5% CO_2.

Statistical Analysis

Data was analyzed using GraphPad Prism (ver. 5.0d). Significance was assessed using the Student's t test unless otherwise indicated in the figure legends. P-values less than 0.05 were considered significant.

Acknowledgements

The authors would like to thank members of the Coombes Lab for helpful discussions. We would like to thank Dr. Cory Hogaboam for the anti-CXCL9 and anti-CXCL10 antibodies, Dr. Bruce Vallance for helpful guidance with the *C. rodentium* staining protocols, and McMaster Histology Services for their assistance with histological staining. 82704

Figure 3.1. *Citrobacter rodentium* is sensitive to CXCL9-directed bacterial killing. **(A)** Dose response survival to increasing concentrations of CXCL9. Wild type *C. rodentium* was exposed to the various concentrations of CXCL9 for 2 h and survival was expressed as a percentage compared to buffer-only controls. Data are the means with standard error from 3 experiments. **(B)** *C. rodentium* time kill curves were plotted in response to 0.39 μg/ml CXCL9. Data are plotted as a percent survival compared to buffer controls without chemokine addition. Data are the means with standard error from 3 experiments. **(C)** PhoP-PhoQ is required for partial resistance to CXCL9-directed killing. The indicated strains were incubated with CXCL9 or α-defensin and survival was determined by viable colony counting. Data are the means with standard error from 3 experiments. **(D)** Anti-CXCL9 antibody blocks the killing activity of CXCL9. *C. rodentium* was incubated with CXCL9 in the presence of either PBS, IgG control, or anti-CXCL9 antibody. CXCL9-specfic antibody blocked 100% of the killing activity. Data are the means with standard error from 3 experiments. **(E)** Membrane permeability in response to CXCL9 or the membrane-disrupting antimicrobial peptide polymyxin B was measured by ANS release. Arrow indicates time of CXCL9 or antimicrobial peptide injection. Data is representative of 3 independent experiments.

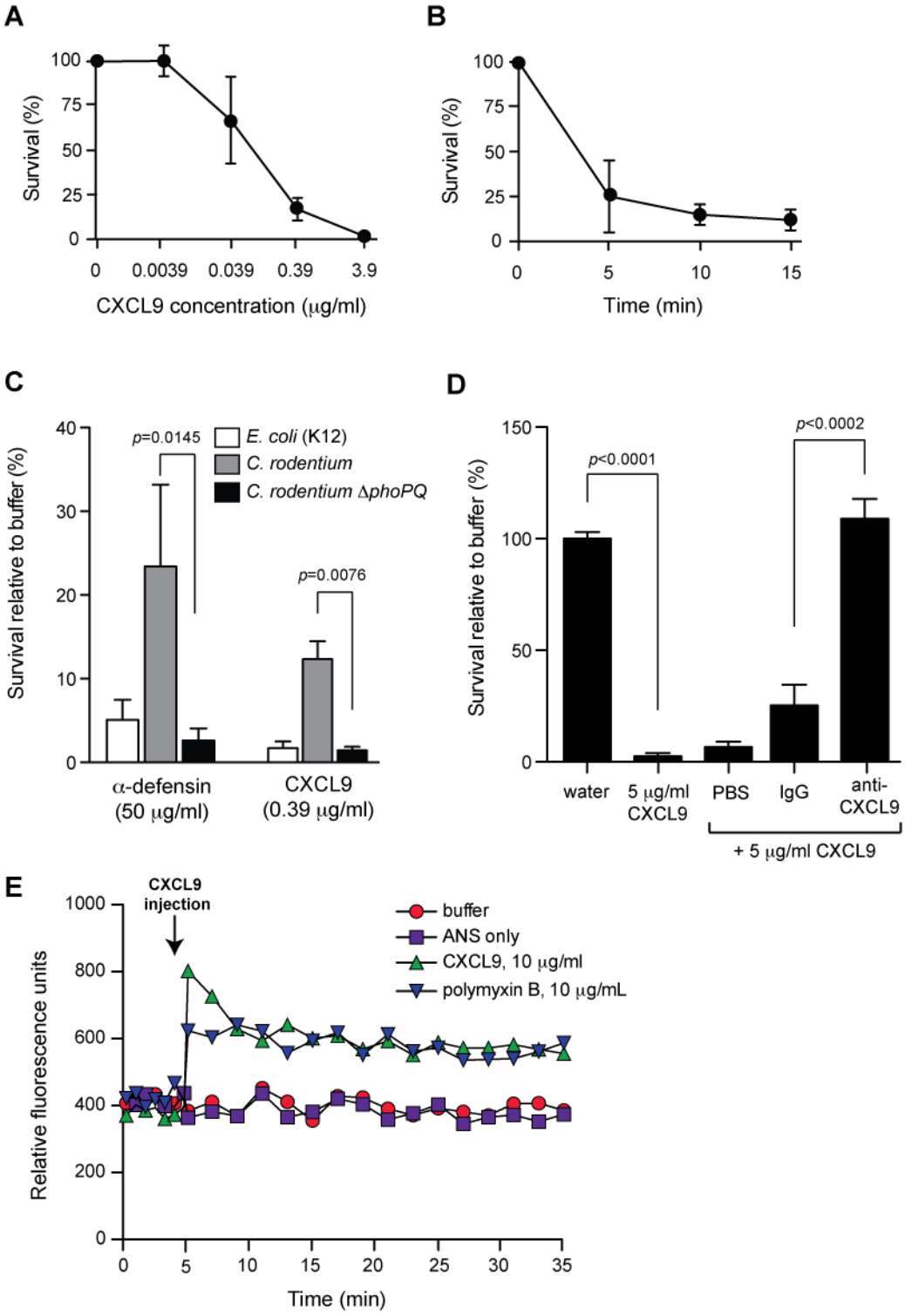
A
Survival (%)
100
75
50
25
0
0
0.0039
0.039
0.39
3.9
CXCL9 concentration (μg/ml)
B
Survival (%)
100
75
50
25
0
0
5
10
15
Time (min)
C
Survival relative to buffer (%)
40
30
20
10
0
p=0.0145
p=0.0076
E. coli (K12)
C. rodentium
C. rodentium ΔphoPQ
α-defensin (50 μg/ml)
CXCL9 (0.39 μg/ml)
D
Survival relative to buffer (%)
150
100
50
0
p<0.0001
p<0.0002
water
5 μg/ml CXCL9
PBS
IgG
anti-CXCL9
+ 5 μg/ml CXCL9
E
CXCL9 injection
Relative fluorescence units
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
Time (min)
buffer
ANS only
CXCL9, 10 μg/ml
polymyxin B, 10 μg/mL

Figure 1

Figure 3.2. Loss of CXCL9 results in increased *C. rodentium* burden and worsened host outcome. (**A**) Rag1$^{-/-}$ mice were infected with *C. rodentium* and administered control rabbit IgG, anti-CXCL9 antibody, or anti-CXCL10 antibody. Survival was monitored for 15 days. Data are from 2 independent experiments. * P <0.05 (Gehan-Breslow-Wilcox) compared to IgG control. **(B)** Rag1$^{-/-}$ mice were infected as above and viable *C. rodentium* was determined in fecal pellets. Data are the means with standard errors from 5 animals. * P <0.05 compared to IgG control. **(C)** Representative images of gross pathology in the cecum and colon of Rag1$^{-/-}$ mice infected with *C. rodentium* for 10 days. Note increased water content in mice depleted of CXCL9, emptied and shrunken cecum and constricted colon. Arrowhead indicates hematoma, which was common in infected CXCL9-depleted animals. **(D)** Water content in fecal output was determined at day 10 after infection. Data is pooled from three separate experiments (9 animals) *P<0.05.

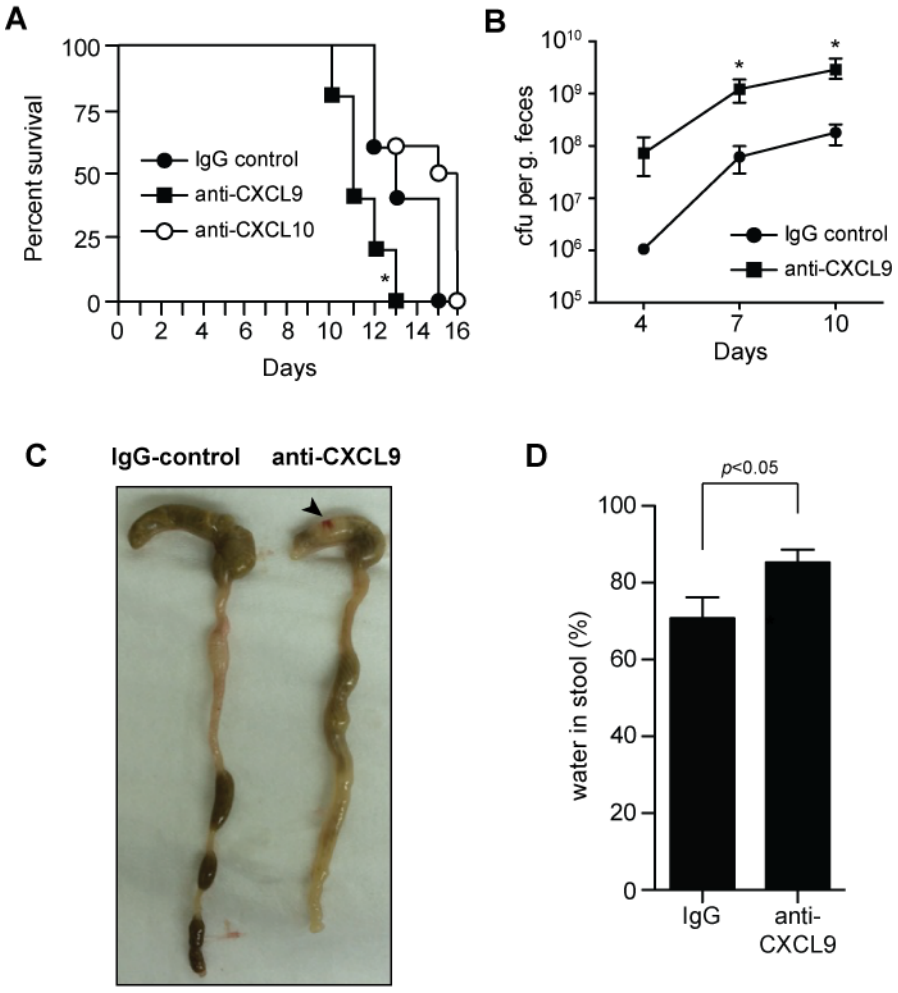
A
Percent survival
100
75
50
25
0
IgG control
anti-CXCL9
anti-CXCL10
*
0 2 4 6 8 10 12 14 16
Days
B
cfu per g. feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
*
*
IgG control
anti-CXCL9
4 7 10
Days
C
IgG-control
anti-CXCL9
D
p<0.05
water in stool (%)
100
80
60
40
20
0
IgG
anti-CXCL9

Figure 2

Figure 3.3. Mice depleted of CXCL9 exhibit greater pathology. (A) Rag1$^{-/-}$ mice were infected with *C. rodentium* and administered IgG control antibody or anti-CXCL9 antibody. Representative histopathology images (200x) of the distal colon are shown from animals infected for 10 days. **(B)** Quantification of pathology in colon. Data is pooled from two experiments, n=6 per group. **(C)** Localization of *C. rodentium* in C57BL/6 colonic tissue by immunohistochemistry. *C. rodentium* in the distal colon from C57BL/6 mice infected for 10 days was localized using an anti-*C. rodentium* antibody. Images (100x) are representative of two separate experiments, n=6 per group. **(D)** Localization of *C. rodentium* in IFN-γ$^{-/-}$ mice by immunohistochemistry. *C. rodentium* in the distal colon from IFN-γ$^{-/-}$ mice infected for 10 days was localized using an anti-*C. rodentium* antibody. Images (200x) are representative of two separate experiments, n=5 per group.

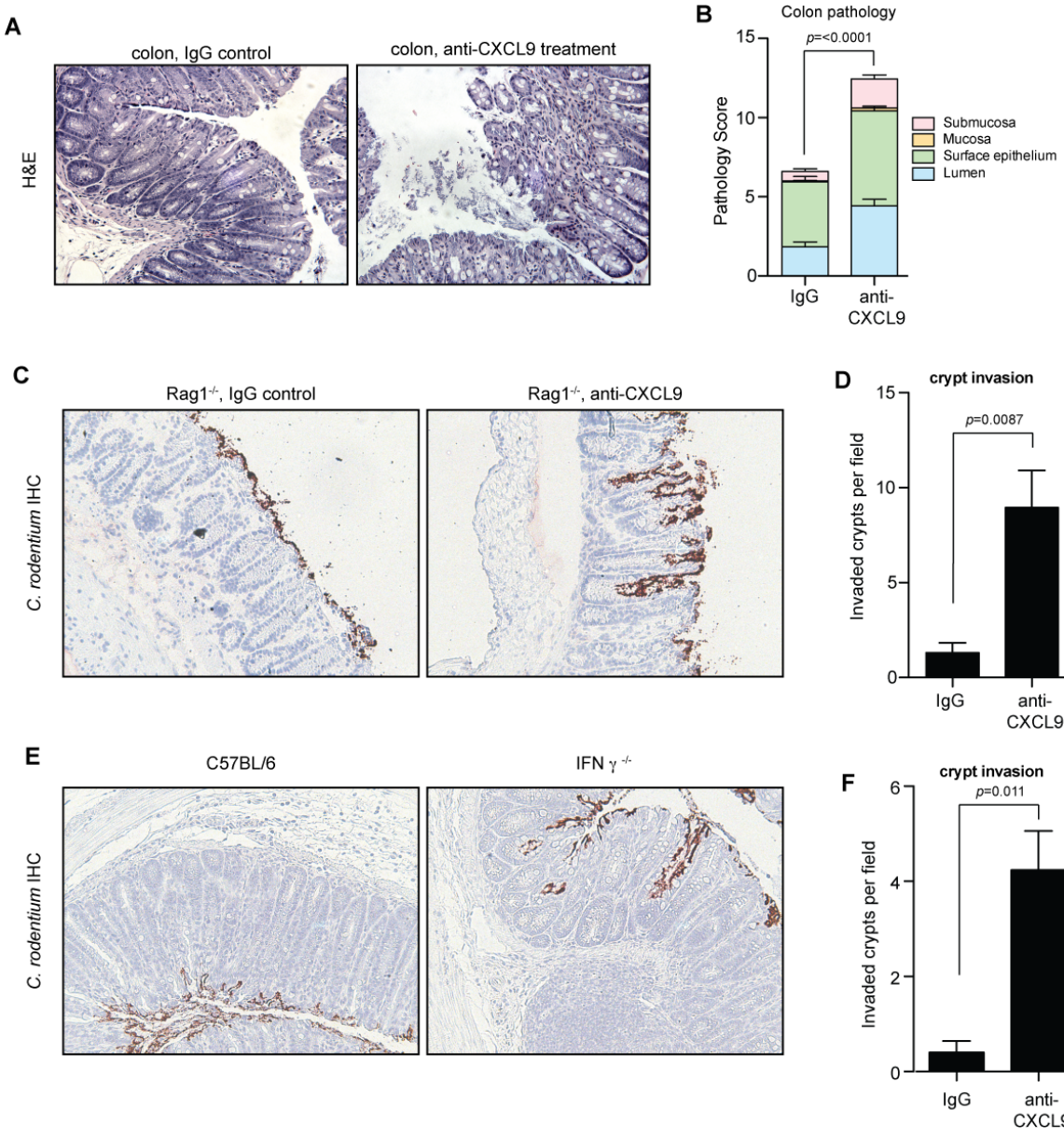
A
colon, IgG control
colon, anti-CXCL9 treatment
H&E
B
Colon pathology
p=<0.0001
Pathology Score
15
10
5
0
Submucosa
Mucosa
Surface epithelium
Lumen
IgG
anti-CXCL9
C
Rag1-/-, IgG control
Rag1-/-, anti-CXCL9
C. rodentium IHC
D
crypt invasion
p=0.0087
Invaded crypts per field
15
10
5
0
IgG
anti-CXCL9
E
C57BL/6
IFN γ -/-
C. rodentium IHC
F
crypt invasion
p=0.011
Invaded crypts per field
6
4
2
0
IgG
anti-CXCL9

Figure 3

Figure 3.4. CXCL9-related phenotypes during *C. rodentium* infection are independent of CXCR3. (A) CXCR3$^{-/-}$ mice were infected with *C. rodentium* and tissue-associated bacterial burden was assessed on day 10 after anti-CXCL9 treatment, or control IgG treatment. Data are the means with standard error from 3 experiments. **P*<0.05. **(B)** Pathology scores in the distal colon of CXCR3$^{-/-}$ mice infected with *C. rodentium.* **(C)** Representative H&E-stained tissue sections (200x). Images are representative from 2 experiments, n=4 per group. **(D)** Localization of *C. rodentium* in CXCR3$^{-/-}$ mice with or without CXCL9 depletion. Immunohistochemistry images (200x) representative of 2 experiments, n=4 per group.

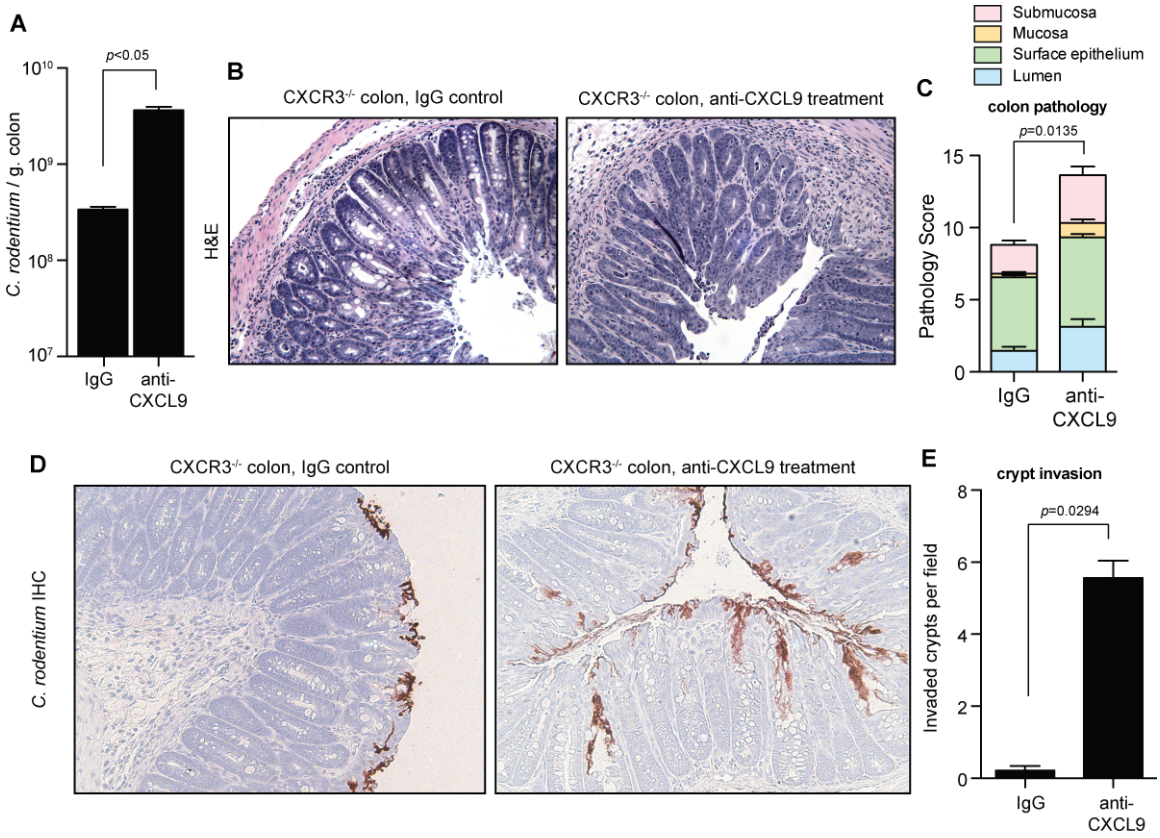
A
p<0.05
C. rodentium / g. colon
IgG
anti-CXCL9
B
H&E
CXCR3-/- colon, IgG control
CXCR3-/- colon, anti-CXCL9 treatment
C
Submucosa
Mucosa
Surface epithelium
Lumen
colon pathology
p=0.0135
Pathology Score
IgG
anti-CXCL9
D
C. rodentium IHC
CXCR3-/- colon, IgG control
CXCR3-/- colon, anti-CXCL9 treatment
E
crypt invasion
p=0.0294
Invaded crypts per field
IgG
anti-CXCL9

Figure 4

Figure 3.5. Macrophages require NK cells and IFNγ for optimal CXCL9 production. **(A)**. Bone marrow-derived macrophages (BMDM), or dendritic cells (BMDC) were incubated for 24 hours at 37°C with either 1 ng/mL IFNγ, and/or heat killed (hk) *C. rodentium*. CXCL9 release was determined from cell culture supernatants by ELISA. Data is the mean with standard error from three experiments. **(B)**. Co-culture of BMDM or BMDC with NK cells leads to increased release of CXCL9. Data is the mean with standard error from three experiments. (**C and D**). $5x10^4$ BMDM, or BMDC from C57BL/6 (WT) or $IFN\gamma^{-/-}$ were incubated for 24 hours at 37°C with C57BL/6 (WT) or $IFN\gamma^{-/-}$ NK cells, hk *C. rodentium*, or media alone, as indicated. Data is the mean with standard error from three experiments. (**E)**. CXCL9 expression was determined from cecal or colonic explant supernatants from *Citrobacter*-infected C57BL/6 (WT), or $IFN\gamma^{-/-}$ mice, day 10 p.i. Explant data is from 5 separate animals per group. Significance was determined using a Student's t test.

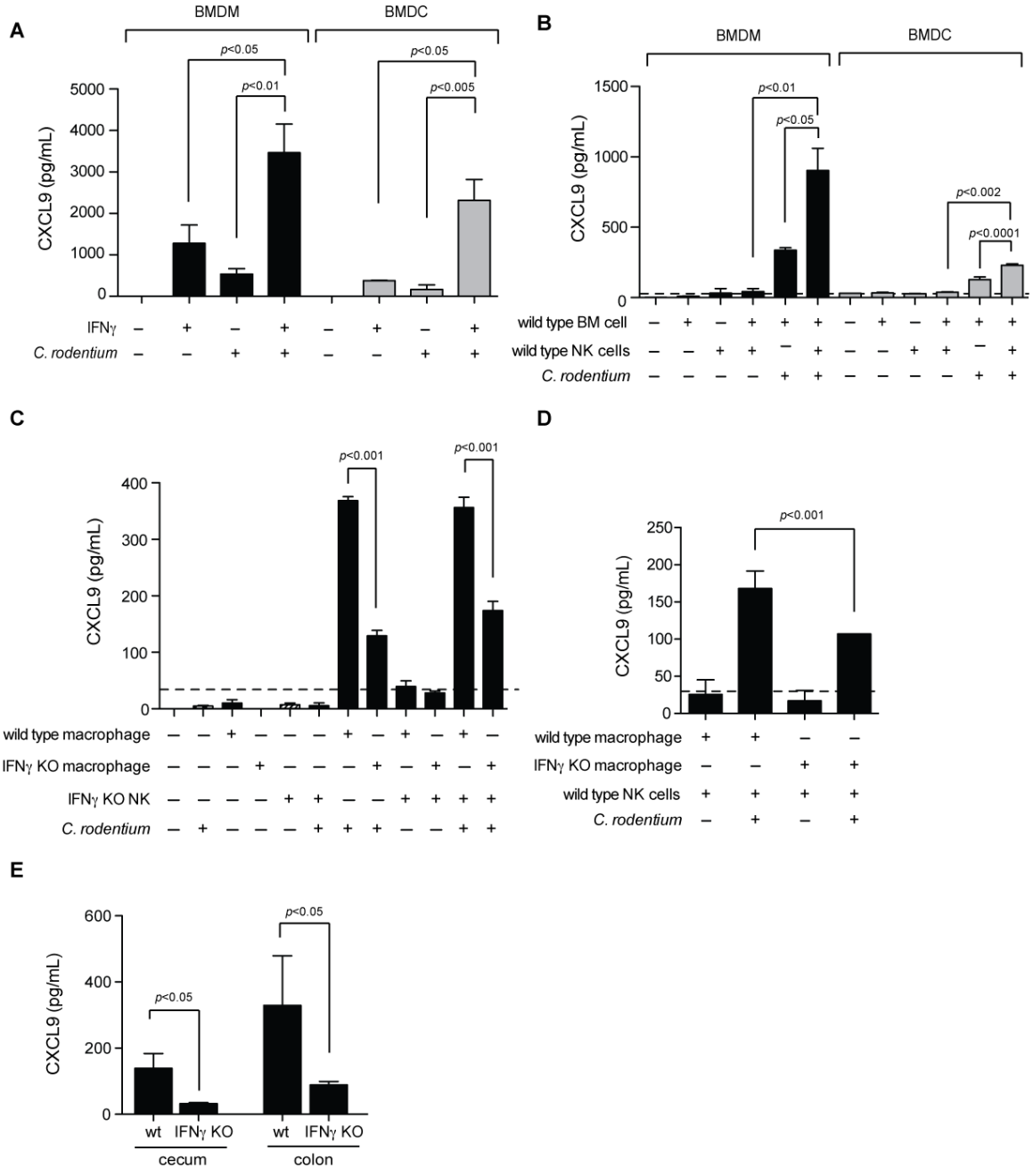
A
BMDM
BMDC
p<0.05
p<0.01
p<0.05
p<0.005
CXCL9 (pg/mL)
5000
4000
3000
2000
1000
0
IFNγ
C. rodentium
B
BMDM
BMDC
p<0.01
p<0.05
p<0.002
p<0.0001
CXCL9 (pg/mL)
1500
1000
500
0
wild type BM cell
wild type NK cells
C. rodentium
C
p<0.001
p<0.001
CXCL9 (pg/mL)
400
300
200
100
0
wild type macrophage
IFNγ KO macrophage
IFNγ KO NK
C. rodentium
D
p<0.001
CXCL9 (pg/mL)
250
200
150
100
50
0
wild type macrophage
IFNγ KO macrophage
wild type NK cells
C. rodentium
E
p<0.05
p<0.05
CXCL9 (pg/mL)
600
400
200
0
wt
IFNγ KO
wt
IFNγ KO
cecum
colon

Figure 5

Fig. 3.S1. *In vivo* depletion of CXCL9 results in loss of T cell homing. (A) Uninfected $Rag1^{-/-}$ mice were injected with control rabbit IgG or anti-CXCL9 antibody. Fecal pellets were collected two days after the second injection and total rabbit IgG was determined by ELISA. **(B)** C57BL/6 mice were infected with *C. rodentium* and given either control rabbit IgG or anti-CXCL9 antibody. The number of CD3+ cells in the distal colon was quantified by immunohistochemical staining using an Image J script. **(C)** CD3+ immunohistochemistry images (200x) representative of 2 experiments.

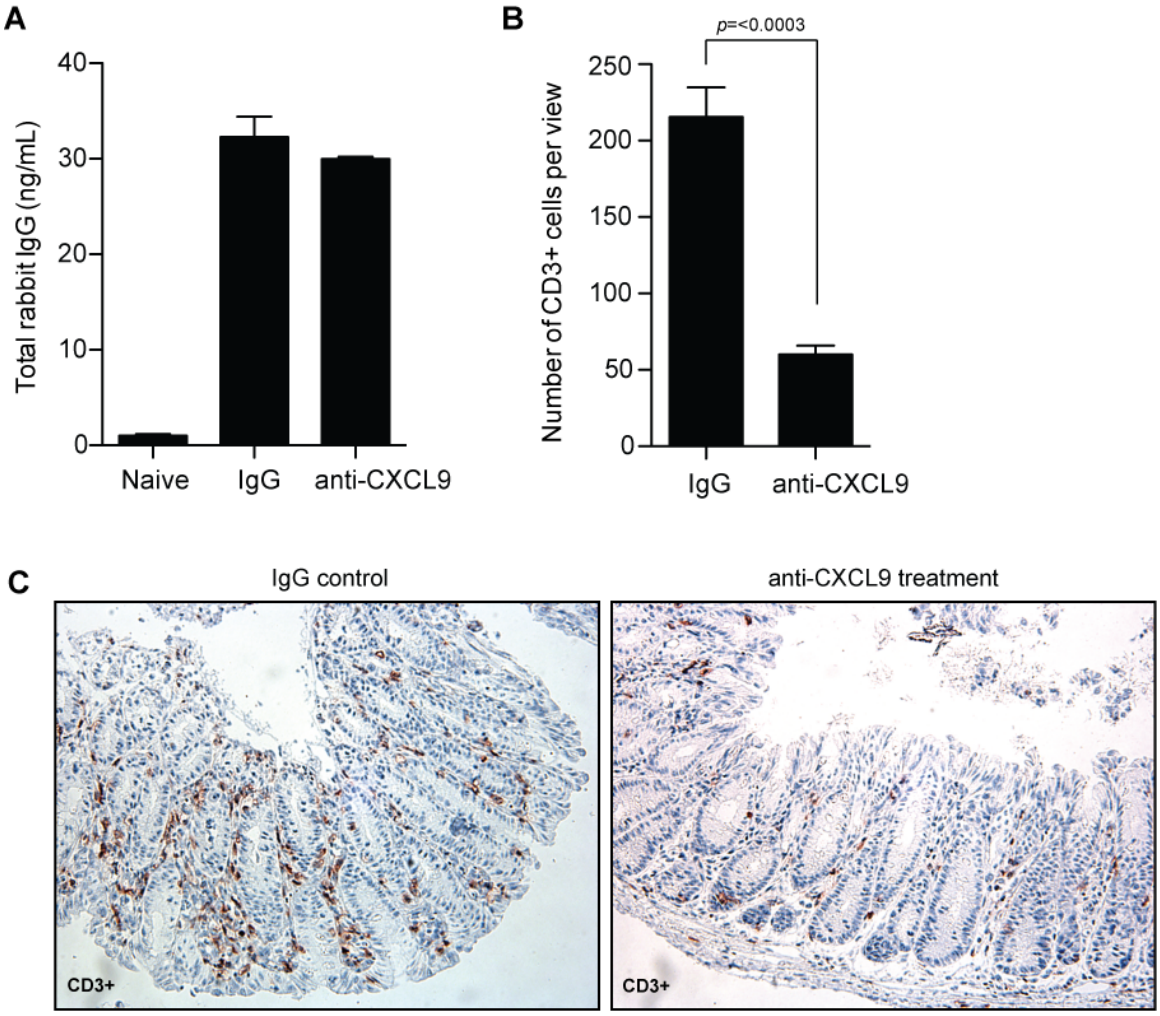
A
Total rabbit IgG (ng/mL)
40
30
20
10
0
Naive
IgG
anti-CXCL9
B
p=<0.0003
Number of CD3+ cells per view
250
200
150
100
50
0
IgG
anti-CXCL9
C
IgG control
anti-CXCL9 treatment
CD3+
CD3+

Figure S1

Fig. S2. No significant changes to cytokine levels upon CXCL9-depletion *in vivo*. Six to eight week old Rag1$^{-/-}$ mice were infected with *C. rodentium* for 10 days and given either anti-CXCL9 antibody or control rabbit IgG. Cytokines were measured by ELISA from the supernatants of colonic explants. All data pooled from two separate experiments, n=6 per group.

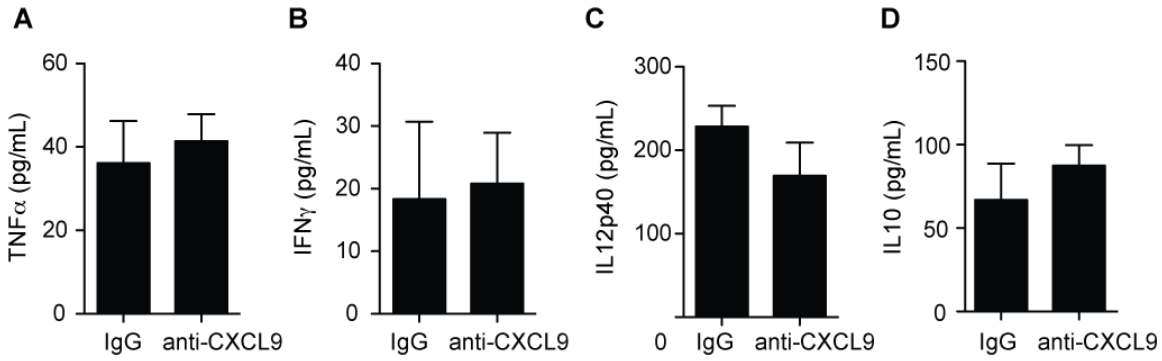
A
B
C
D
TNFα (pg/mL)
IFNγ (pg/mL)
IL12p40 (pg/mL)
IL10 (pg/mL)
60
40
20
0
30
10
300
200
100
150
50
IgG
anti-CXCL9

Figure S2

Fig. 3.S3. Cecal histology in CXCL9-depleted animals. Rag1$^{-/-}$ mice were infected with *C. rodentium* and administered either anti-CXCL9 antibody or control rabbit IgG. **(A)** Representative H&E-stained sections taken from the cecum (200x). Pathology scores in the cecum are quantified in **(B)**. Images and data are pooled from two experiments, n=6 per group. **(C)** Localization of *C. rodentium* by immunohistochemical staining. Images (200X) are representative of 2 experiments, n=6 per group.

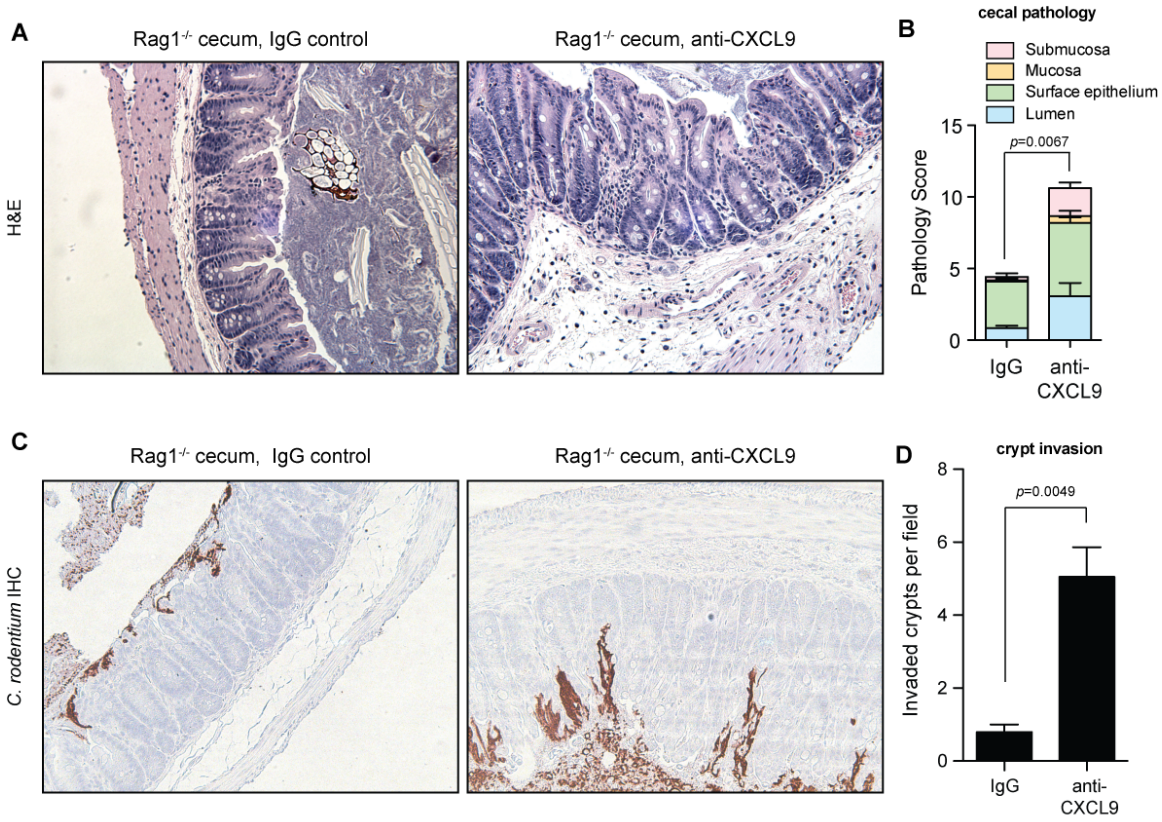
A
Rag1-/- cecum, IgG control
Rag1-/- cecum, anti-CXCL9
H&E
B
cecal pathology
Submucosa
Mucosa
Surface epithelium
Lumen
p=0.0067
Pathology Score
15
10
5
0
IgG
anti-CXCL9
C
Rag1-/- cecum, IgG control
Rag1-/- cecum, anti-CXCL9
C. rodentium IHC
D
crypt invasion
p=0.0049
Invaded crypts per field
8
6
4
2
0
IgG
anti-CXCL9

Figure S3

Fig. 3.S4. Cecal histology in CXCR3$^{-/-}$ animals containing CXCL9, or depleted of CXCL9. CXCR3$^{-/-}$ mice were infected with *C. rodentium* and administered either anti-CXCL9 antibody or control rabbit IgG. **(A)** Representative H&E-stained sections taken from the cecum (200x). Pathology scores in the cecum are quantified in **(B)**. Images and data are pooled from two experiments, n=4 per group. **(C)** Localization of *C. rodentium* by immunohistochemical staining. Images (200X) are representative of 2 experiments, n=4 per group.

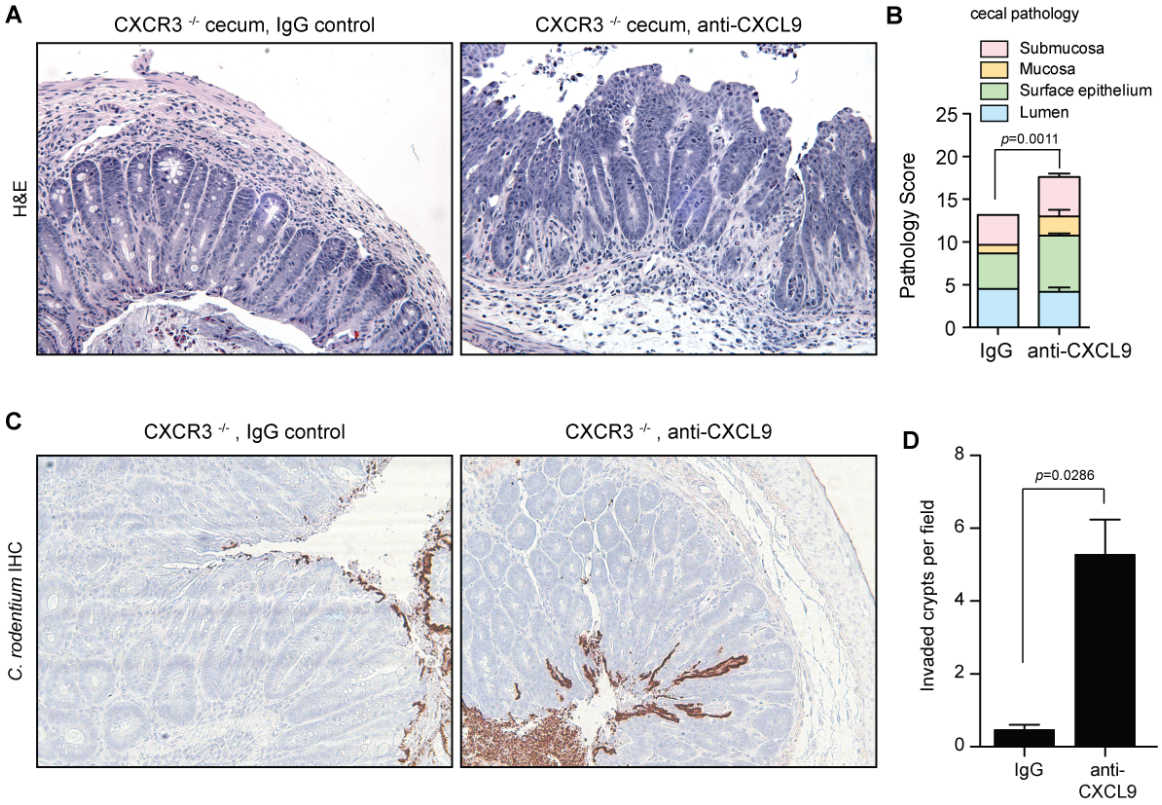
A
CXCR3 -/- cecum, IgG control
CXCR3 -/- cecum, anti-CXCL9
H&E
B
cecal pathology
Submucosa
Mucosa
Surface epithelium
Lumen
p=0.0011
Pathology Score
25
20
15
10
5
0
IgG
anti-CXCL9
C
CXCR3 -/- , IgG control
CXCR3 -/- , anti-CXCL9
C. rodentium IHC
D
p=0.0286
Invaded crypts per field
8
6
4
2
0
IgG
anti-CXCL9

Figure S4

Chapter Four

Discussion

Chapter Four – Discussion

Overview of Major Findings

The studies presented here provide insight into the role of NK cells in host defense against extracellular enteric pathogen, *Citrobacter rodentium*. Previous to the commencement of this study, it was well established that *C. rodentium* induced a robust Th1 response, and that IFNγ was critical for controlling bacterial burden at initial sites within the colon and systemic sites, as well as in clearance of the bacterium and limiting intestinal pathology [72,90]. Later, IFNγ-producing CD4+ T cells, independent of B cells, were found to be important for mediating all three of these protective effects [136]. Yet, it was still unknown what innate immune cells were important for generating these responses within the *Citrobacter* infection model, or if other IFNγ-producing cells participated in direct host protection.

Generation of Th1 CD4+ T cells from naïve T cells is dependent upon presentation of foreign peptide by antigen presenting cells, such as DCs, within the draining lymph node [225,226,315]. NK cells, however, also aid in development of Th1 CD4+ T cells through their ability to home to the lymph node, and provide an essential source of IFNγ, which is critical for the development of these cells [231,316]. In accordance with this, many have observed an important role for NK cells in directing Th1

responses to a variety of intra- and extracellular pathogens; thus, leading us to question whether NK cells could play a similar role in *C. rodentium* infections [240-242,267].

Indeed, as shown in Chapter 2, we found that CD3-NK1.1+IFNγ+ innate lymphocyte cells, reminiscent of NK cells, were capable of becoming activated, and expanding in responses to *C. rodentium* infection (Figures 2.1 and 2.S1). These cells were shown to traffic to the mesenteric lymph nodes, and animals depleted of these cells had delayed Th1 CD4+ T cell recruitment in the colon, as well as delayed bacterial clearance, decreased levels of pathogen-specific IgG, and a greater degree of intestinal pathology, compared to wild type controls (Figures 2.1, 2.2, 2.4, and 2.5). While we were not able to explicitly attribute these findings to NK cells, and not ILC1s, which share similar CD3-NK1.1+IFNγ+ phenotype, we did find that unlike the NK cell population, there was limited expansion of NK1.1+ ILC1s upon infection (Figure 2.1 and 2.S1). In addition, ILC1s have been linked to deleterious effects on intestinal pathology in models of intestinal inflammation, and their depletion led to the ameliorating of this phenotype [317,318]. Therefore, it is likely that it was the loss of NK cells, and not ILC1s, in our model, which led to the increased degree of intestinal pathology, as well as delayed Th1 responses. However, further experimentation will be necessary to more completely divide the roles of ILC1s and NK cells in *Citrobacter rodentium* infections.

In addition to shaping adaptive responses to A/E pathogens, we speculated that IFNγ-producing NK cells also played a role in innate defenses, independent of B and T cells as had been previously shown in Rag1-/- mice [143]. Beyond observations for a role for NK cells in promoting survival in mice lacking T and B cells, we had observed in our

own studies that mice depleted of NK1.1+ cells exhibited diminished levels of CXCL9, a CXCR3-binding chemokine (Figure 2.3). At the time, we attributed not only loss of NK1.1+ cells trafficking to the lymph node, but also decreased levels of CXCR3-binding chemokines, to a delay in Th1 responses in NK1.1-depleted animals. However, in recent years, due to their similarity in structure and charge, multiple chemokines, including CXCL9, have been shown to exhibit direct antimicrobial action *in vitro* against both Gram-negative, and Gram-positive bacteria [100,101,105,319-322]. Thus, we speculated that IFNγ-producing NK cells could potentially aid in the induction of antimicrobial chemokine expression in the infected colon, and that these IFNγ-induced chemokines may play important roles in host defense against A/E pathogens (Chapter 3). Indeed, we were able to observe *in vitro* CXCL9-directed antimicrobial activity against *Citrobacter*, and exposure of the bacteria to CXCL9 resulted in increased bacterial membrane permeability (Figure 3.1). The antimicrobial activity of CXCL9 could be blocked through addition of anti-CXCL9 antibodies (Figure 3.1). Due to their quintessential nature in homing of CXCR3+ T cells to the site of infection, we chose to investigate the potential *in vivo* antimicrobial nature of CXCL9 in Rag1-/- mice [323]. Antibody-directed neutralization of CXCL9 led to accelerated mortality in weanling mice, increased bacterial burden, and greater degree of intestinal pathology (Figure 3.2 and 3.3). We also observed a deeper penetration of *Citrobacter* within the colonic crypts (Figure 3.3), a phenomenon also observed in mice deficient in the expression of antimicrobial peptide, CRAMP [93]. Despite previous reports identifying a multitude of sources capable of CXCL9 expression, such as monocytes/macrophages, CD11c+ cells, neutrophils, and

epithelial cells, we observed that bone marrow-derived macrophages were capable of robust CXCL9 expression (Figure 3.5) [281,324-329]. *In vitro* cell culture assays revealed that IFNγ from both NK cells and macrophages were critical for optimal CXCL9 expression in response to *C. rodentium* (Figure 3.5). These findings suggest that both NK cells and macrophages likely participate in directing the potential direct antimicrobial activity of CXCL9 in *C. rodentium* infections, and reveal a potential innate protective role for NK cells within the infected colon.

Remaining Questions: Generation of Th1 Responses in Citrobacter

In addition to teasing apart the separate roles for ILC1s, and NK cells in mediating the observed phenotypes in *Citrobacter*-infected NK1.1-depleted mice, there is also a need to further examine the potentially essential role of NK cells trafficking to the mesenteric lymph nodes in generation of Th1 responses in *C. rodentium* infection. While we provided a correlative observation between NK cell trafficking to the draining lymph node and a robust Th1 response to *C. rodentium*, to concretely state this is the case, however, we would need to monitor the differentiation and proliferation of these responses in the lymph node, and confirm that this activity is delayed in NK1.1-depleted infected animals. This could be achieved by examining the expression level of T-bet, the essential Th1 transcription factor, within proliferating CD4+ T cells. As well, as we observed only a delay and not a complete elimination in the generation of CD4+ Th1 responses, we speculated that there may be a less efficient secondary source of IFNγ

trafficking to or within the lymph node, or that differentiating CD4+ T cells can provide sufficient IFNγ to produce this delayed response. Therefore, in addition to monitoring T-bet expression in CD4+ T cells within the mesenteric lymph nodes, we could also potentially identify what, if any, secondary cellular sources of IFNγ are present and if they are indeed necessary for generating the delayed Th1 response we observe in NK1.1-depleted animals.

In addition to understanding if secondary sources of IFNγ are required for the delayed Th1 responses, it would also be of interest to determine what is driving activation of NK cells at the site of infection; whether these responses are due to direct sensing of *Citrobacter*, or through activation by other accessory immune cells. Recent evidence has shown that NK cells can be directly cytotoxic towards *C. rodentium*, implicating that NK cells can become directly activated by these bacteria [189]. In addition to being directly activated by *Citrobacter*, it is likely that other innate immune cells play an important role in the activation of NK cells. In particular, dendritic cells have been shown, primarily through expression of IL12 and IL15, to amplify NK cell activation, and IFNγ production by these cells [330,331]. However, both neutrophils and monocytes/macrophages have also been known to regulate NK cell responses [154,155,332]. Further examination of NK cell activation in response to *Citrobacter* both *in vitro* and *in vivo*, and the necessity for accessory immune cells is necessary to better understand how NK cells respond to A/E pathogens.

Aside from the potential role dendritic cells and macrophages play in NK cell activation, their role in the generation of Th1 responses is a subject of further interest.

DCs are the predominate APC known to be critical for the development, and amplification of Th1 CD4+ T cell responses within the draining lymph nodes [226,333,334]. Recently, DCs have been shown to be essential for priming CD4+ T cells in *Citrobacter* infected mice, as depletion of these cells resulted in a significant decrease in activated CD4+ T cells. Alternatively, monocytes/macrophages were shown to be important for maintenance of IFNγ production within activated CD4+ T cells, but not in generation of these activated CD4+ T cells themselves [220]. Macrophages appear to mediate these effects through expression of IL12, similar to observations made with *Listeria monocytogenes* infections where infected macrophages were a critical source of IL12 essential for maintaining Th1 CD4+ T cell activation at the primary site of infection [220,228]. Within the colon, both DCs and macrophages have been shown to be important sources of IL12, a cytokine not only known to be essential for survival, and virulence of *Citrobacter* infections, but also in driving Th1 responses [220,229,254,335]. However, depletion studies revealed that due to their increased abundance compared to DCs, macrophages are the predominant source of IL12 within the colon [220]. Thus, while DCs are critical for priming Th1 CD4+ T cells, macrophages are essential for maintaining their IFNγ production within the primary site of infection.

In addition to an essential nature of IL12 for maintaining Th1 CD4+ T cell IFNγ production, it was previously thought further antigen presentation of their cognate antigenic type at the mucosal surface was also essential for maintaining Th1 phenotype. Recent evidence, however, has shown that CD4+ T cells work primarily through a bystander effect where their interaction with only a small number of infected cells, or

APCs is sufficient to mediate their responses [336]. Alternatively, a TLR-dependent mechanism of driving Th1 CD4+ T cell activation has recently been observed, which functions independently of presented antigen, but is dependent on IL-18 expression. This particular mechanism is important particularly with the intracellular pathogen, *Salmonella enterica* serovar Typhimurium [337,338]. However, it is unclear whether this is a common mechanism utilized in host defense of extracellular pathogens within the gut.

Remaining Questions: Antimicrobial CXCL9

The observation of the potent antimicrobial activity of CXCL9, and other cationic chemokines, has brought into question the precise role of these proteins within host defense to bacterial pathogens (discussed in Chapter 3). A comparison of gene expression profiles from uninfected versus *Citrobacter*-infected colons revealed that genes encoding for antimicrobials peptides and chemokines are among the most highly activated upon infection suggesting these peptides/proteins play a predominate role in host defense. A large percentage of elevated chemokine genes expressed in *Citrobacter* infected colons were CXC chemokines such as CXCL1, CXCL2, CXCL5, CXCL9, and CXCL10 [281]. While the majority of these, including CXCL9, have been shown to be instrumental in immune cell targeting to the infected site of the colon, it is unknown what potential protective *in vivo* antimicrobial role chemokines, other than CXCL9, may play independent of their chemotactic capabilities [95,281]. Reexamination of antimicrobial function of these chemokines would, like CXCL9, need to be examined independent of

their respective receptors. While our work in Chapter 3 revealed a direct protective role for CXCL9, independent of its receptor, further proof is required to ascertain direct antimicrobial protection by this chemokine. Exogenous delivery of these chemokines into infected mice would be the next step in not only showing direct antimicrobial function, but also potential therapeutic capabilities of CXCL9, and other antimicrobial chemokines. We would then confirm whether these chemokines are capable of mediating similar protective effects as have been attributed to them *in vitro*. In order to properly identify and isolate the direct antimicrobial effects of these exogenous chemokines, these investigations would need to be carried out in mice deficient for both their known receptors and the respective chemokine, and/or with solely the portion of the chemokine known to contain its antimicrobial properties.

Addressing the efficacy of the delivery exogenous chemokines in protection against bacterial pathogens is only part of assessing their future therapeutic potential. Additionally, one must also understand how pathogens develop resistance, and the common mechanisms they employ to do so. Though the majority of cationic antimicrobial chemokines/peptides have been shown to mediate their effects through broad-spectrum mechanisms, thus making it difficult for bacteria to evolve resistance to these antimicrobials, several pathogens have evolved mechanisms to combat their antimicrobial actions. Currently, several bacteria have been shown to possess resistance against antimicrobial chemokines. They predominately achieve this through prevention of their association with the bacterial outer membrane either by secretion of proteins capable of binding the chemokine, or bacterial binding of large host proteins such as albumin

[311,339]. Alternatively, resistance can also be mediated through expression of proteinases that cleave the chemokine, or AMP [277]. Likewise, A/E pathogens also possess a membrane-associated protease, known as CroP in *C. rodentium*, which confers resistance to AMPs, such as the cathelicidin, CRAMP [340,341]. However, this protease does not offer ubiquitous protection, as *Citrobacter* is susceptible to other AMPs such as polymyxin B [340]. Examination of membrane permeability dynamics upon exposure of *C. rodentium* to CRAMP, Le Sage *et al.* observed a delayed increase in permeability, whereas exposure to polymyxin B (AMP not digested by CroP) exhibited an immediate increase in permeability, similar to our observations with polymyxin B and CXCL9 [340]. Thus, due to similar membrane permeability dynamics to polymyxin B, and the highly potent actions of CXCL9 against *Citrobacter*, it is unlikely CroP plays an important protective role against this chemokine. However, it would be of interest to determine what, if any, role it plays in bacterial defense against other antimicrobial chemokines.

In addition to AMP resistance mediated by expression of membrane-associated proteases, many pathogens both sense and mediate resistance via the PhoP-PhoQ two-component regulatory system. Indeed, in Chapter 3, we found that *C. rodentium* does appear to possess a modest level of resistance to CXCL9, which was lost in a Δ*phoPQ C. rodentium* mutant. PhoQ is a sensor kinase that becomes activated upon exposure to low Mg+, low pH, and/or sub-MIC concentrations of α-helical AMPs [342-344]. Activation of PhoQ, and therefore PhoP, promotes protection against AMPs through activation of genes important for modification of lipid A, a component of bacterial outer membrane lipopolysaccharide (LPS), resulting in both strengthening of the bacterial outer

membrane, and reduction of it's overall negative charge [345,346]. Mechanisms of PhoPQ-mediated resistance to AMPs has been primarily studied in *S.* Typhimurium, a bacterium that possesses multiple genes necessary for lipid A modifications [346,347]. *Citrobacter,* however, is lacking the majority of these known lipid A modification genes, and only possesses PhoPQ-regulated *pagP* and PmrAB-regulated genes *pmrC* and *cptA*. PagP regulates palmitate transfer to lipid A, while the latter genes control addition of phosphoethanolamine [25,348,349]. While PmrAB-regulated genes are important for maintenance of outer membrane integrity in *C. rodentium*, these genes, unlike in *S.* Typhimurium, are not a PhoPQ-regulated gene cluster; thus, any resistance observed in our Δ*phoPQ C. rodentium* mutant was likely dependent up PagP-directed lipid A modifications, or in an as of yet undetermined protective function of PhoPQ [347,349]. Further investigation into the LPS modifications directing the modest resistance of *C. rodentium* to various AMPs is warranted, and would provide clarity to A/E pathogen mediated resistance to antimicrobial chemokines.

Concluding Remarks

The studies presented here validate the hypothesis that NK cells function in IFNγ-mediated innate and adaptive host defense. NK, and/or ILC1s, were found be instrumental in stimulating robust Th1 CD4+ T cell responses to the *Citrobacter*-infected colon. Additionally, NK cells were found to play a potential role in innate host defense through promotion of CXCR3-independent CXCL9 activity within the colon, likely

through IFNγ-directed activation of macrophages. These observations provide evidence for how NK cells attribute to overall IFNγ-mediated host defense to extracellular enteric pathogen, *C. rodentium*.

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