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The role of chemical signaling and gut mucosal immunity during *Citrobacter rodentium* infection

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"*If you can dream it, you can do it*."

Walt Disney

Abstract

The human pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are transmitted by the fecal-oral route and cause severe diseases worldwide. Due to their potential to induce pathological lesions in the gut epithelium, they are commonly known as *attaching and effacing* pathogens (A/E pathogens). To date, effective strategies to manage EPEC and EHEC infections beyond standard supportive measures are lacking. Therefore, preclinical studies to better understand the mechanisms of infection of EPEC and EHEC are required. Because these bacteria are poorly pathogenic in mice, *Citrobacter rodentium* has been broadly used as an alternative *in vivo* model pathogen to study EHEC and EPEC disease. Similarly, to human pathogens, *C. rodentium* causes A/E lesions in mice without requiring antibiotic pretreatment to establish infection.

Studies have shown that the membrane-bound histidine sensor kinase QseC promotes A/E pathogen colonization *in vivo*. However, whether QseC signaling is essential for A/E pathogens to resist and evade immune responses to establish disease remains unknown. On the host side, interleukin IL-22 is critical to resolve *C. rodentium*-mediated disease, although the exact mechanism(s) of IL-22-mediated protection are not well understood. Here, we aimed to investigate the role of chemical signaling through the QseC sensor for establishing disease and resistance to the host immune response. Furthermore, we studied the downstream mechanisms that mediate the mucosal immune protection conferred by IL-22 during *C. rodentium* infection.

Our data suggest that the QseC sensor is directly involved in triggering intestinal inflammation and promoting transmissible colonic crypt hyperplasia, a hallmark pathological feature of *C. rodentium*-mediated disease. Moreover, QseC-mediated signaling enhanced *C. rodentium*'s resistance to neutrophil antimicrobial responses, resulting in the pathogen's systemic dissemination *in vivo*. We identified changes in the lipopolysaccharide membrane modulated by the QseC sensor as a potential mechanism. Furthermore, we found that the IL-22-mediated resolution of *C. rodentium* infection was independent of the induction of several downstream responses, including calprotectin, Reg3β, Reg3γ, lipocalin-2, and C3. Instead, the susceptibility of IL-22 deficient mice to *C. rodentium* was primarily associated with increased intestinal permeability. Together, our data show that *C. rodentium* depends on chemical signaling mediated by QseC for disease establishment and to resist the immune responses conferred by neutrophils. Upon infection, IL-22 plays an essential role in host protection, which was independent of a single deficiency of either antimicrobial peptide regulated by this cytokine; instead, protection was related to IL-22's regenerative properties by promoting the epithelial

barrier integrity. Overall, our work expands the knowledge of the *in vivo* mechanisms triggered by A/E pathogens during infection.

Keywords: Chemical Signaling, Citrobacter rodentium, Immune response, Interleukin-22.

Resumo

Escherichia coli enteropatogênica (EPEC) e *E. coli* enterohemorrágica (EHEC) são patógenos humanos transmitidos pela via fecal-oral que causam doenças graves mundialmente. Devido a lesão que desenvolvem em células epiteliais intestinais, essas bactérias são conhecidas como patógenos *Attaching and Effacing* (patógenos A/E). Até o momento nenhuma estratégia eficaz foi desenvolvida para combater as infecções causadas por EHEC e EPEC, sendo o tratamento restrito apenas a suporte. Portanto, estudos pré-clínicos para melhor entender os mecanismos de infecção de EPEC e EHEC são necessários. Como essas bactérias são pouco patogênicas em camundongos, *Citrobacter rodentium* tem sido utilizada como um patógeno modelo alternativo para estudar *in vivo* a doença causada por EHEC e EPEC, uma vez que causa lesão A/E em camundongos e não requer pré-tratamento com antibióticos para estabelecer a infecção.

Estudos demonstraram que o sensor QseC, presente na membrana de bactérias, beneficia a colonização de patógenos A/E em vivo. No entanto, pouco se sabe se a sinalização através do sensor QseC em patógenos A/E é essencial para evadir e resistir às respostas imunes do hospedeiro para o estabelecimento da doença. Ainda, estudos demonstram que a interleucina-22 é essencial para a resolução da doença causada por *C. rodentium* em camundongos embora o(s) mecanismo(s) exato(s) de proteção mediada por Il-22 ainda necessitem de mais investigação. Desta forma, este trabalho foi desenvolvido com o intuito de investigar a participação da sinalização química através do sensor QseC para estabelecimento da infecção e resistência à resposta imune do hospedeiro, e o mecanismo mediado por IL-22 que confere proteção durante a infecção por *C. rodentium.*

Nossos resultados sugerem que o sensor QseC está diretamente envolvido no desencadeamento da inflamação intestinal e desenvolvimento da hiperplasia transmissível da cripta colônica, sendo esta última, uma característica da doença mediada por *C. rodentium*. Além disso, através da sinalização mediada por QseC, *C. rodentium* tem sua sobrevivência a neutrófilos aumentada, o que pode estar associado com sua disseminação sistêmica *in vivo* que pode ser devido a mudanças na membrana de lipopolissacarídeo modulada pelo sensor QseC. Ainda, nossos resultados sugerem que uma vez estabelecida a infecção, a citocina IL-22 é fundamental para a resolução da doença mediada por *C. rodentium*, uma vez que camundongos deficientes em IL-22 não sobrevivem à infecção. Porém esse fenótipo não é dependente de respostas mediadas pelas proteínas antimicrobianas: calprotectina, REG3, lipocalina-2 e C3, mas pode estar associada à indução de mecanismos de regeneração e integridade do epitélio mediados por IL-22. Juntos, esses resultados demonstram que patógenos A/E

dependem da sinalização química via QseC para resistir à resposta imune mediada por neutrófilos e para o estabelecimento da doença causada por *C. rodentium* em camundongos. Após a infecção, IL-22 desempenha um papel essencial na proteção do hospedeiro, que não depende da resposta isolada de peptídeo antimicrobianos regulados por esta citocina; ao contrário, essa proteção pode estar associada à recuperação do epitélio intestinal mediada por IL-22, promovendo integridade epitelial. De modo geral, nosso trabalho amplia o conhecimento dos mecanismos *in vivo* desencadeados por patógenos A/E durante a infecção.

Palavras-chave: Sinalização química, *Citrobacter rodentium*, Resposta Imune, Interleucina-22

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List of Abbreviations

CXCR2 - *C-X-C Motif Chemokine Receptor 2*

CXCL - *C-X-C Motif Chemokine Ligand*

IL-22 - *Interleukine-22*

Lcn2 - *Lipocalin-2*

REG3 - Islet-derived protein 3

S100A9 - S100 calcium-binding protein

C3 - Complement component 3

NET - *Neutrophil extracellular traps*

% - Percentage

A/E - *Attaching and Effacing*

AI-3 - Autoinducer-3

C. rodentium - Citrobacter rodentium

TCCH - *Transmissible colonic crypt hyperplasia*

CFU - *Colony Forming Unit*

FITC - *Fluorescein isothiocyanate*

OD600 - *Optical density at 600 nm*

EHEC - *Enterohaemorrhagic Escherichia coli*

EPEC - *Enteropathogenic Escherichia coli*

LB - *Lysogeny broth*

LEE - *Locus of Enterocyte Effacement*

LPS - *Lipopolysaccharide*

MOI - *Multiplicity of Infection*

QseC - *Quorum sensing E. coli C (sensor kinase)*

HUS - *Hemolytic uremic syndrome*

STEC - *Shiga Toxin-Producing Escherichia coli*

S. Typhimurium - *Salmonella enterica* serovar Typhimurium

T3SS - *Type III Secretion System*

qRT-PCR - *Quantitative Reverse Transcriptase Polymerase Chain Reaction*

Chapter I: Literature Review

1. Introduction

1.1. The gastrointestinal tract

The gastrointestinal tract (GI tract) is part of the digestive system of humans and other animals that extends from the mouth to the anus and contains the esophagus, stomach, and small and large intestines (1). The gut is a muscular tube composed of the luminal epithelium and the underlying lamina propria and muscularis mucosae layers. In addition to the GI tract, the digestive system also comprises the accessory organs: tongue, salivary glands, pancreas, liver, and gallbladder. Its primary function is the digestion and absorption of food (1,2).

The GI tract is colonized by a collection of bacteria, archaea, viruses, and fungi termed “gut microbiota” (3,4). The gut microbiota is a microbial community estimated to exceed 10^{14} microorganisms that co-evolved to form a mutually beneficial relationship with their host (3,5). The microbial composition differs between hosts and is often influenced by health, age, gender, and diet differences (4). The gut microbiota is fundamental to the host’s health. Its beneficial roles include digesting food and harvesting energy, training and regulating host immunity, strengthening gut integrity, and protecting against pathogens (3,5).

The microbiota is compartmentalized within the host, and its interactions are tightly regulated by multiple layers of physical, chemical, and immunological barriers (6); the intestinal epithelium is a single layer segregating the outer world from the inner body (7,8). It is covered by the mucus layer, which comprises mucins, antimicrobial proteins, and

Immunoglobulin (Ig) A, thereby preventing microorganisms from reaching the epithelial cells (9–12). The IEC monolayer is composed of specialized cell types that differentiate between residing epithelial cells, goblet cells, absorptive enterocytes, enteroendocrine cells, Microfold cells, and Paneth cells (13,14). These cells confer functions that range from nutrient absorption to mucus and antimicrobial peptide production for host protection (14). The underlying lamina propria consists of connective tissue with interspersed immune cells, separating the epithelial layer from the smooth muscle (15,16). The numerous immune cells within this layer have an essential role in homeostasis maintenance and defense against external threats (**Figure 1**). Moreover, Peyer's patches, the draining mesenteric lymph nodes (9,17), which, along with the diffuse lymphoid tissue of the lamina propria, form the gut-associated lymphoid tissue (GALT) that fundamentally contributes to the host's health by conferring protection and tolerance from foreign antigens and microbes alike due to the immune cells that habits this lymphoid tissues (9,17,18).

The dynamic between the host, environmental factors such as diet, and microbiota are complex. Clinical trials in humans have been conducted to understand this dynamic. However, there are limitations in the studies due to practical and ethical reasons. For these matters, mouse models have been extensively used to understand the interaction between intestinal microbiota, gut immune response, and diet within the gastrointestinal tract (19).

1.2. Gut immunity: a fine balance between protection and tolerance

The immune system constitutes a complex network of biological processes that involve protective responses against threats within an organism. Traditionally, the

immune system has been classified into two subsystems: innate and adaptative immunity, although with overlaps. The innate immune response has a limited recognition of molecular patterns. In contrast, the adaptive immune system mounts specific responses by recognizing a wide range repertoire of molecules (20).

The intestinal mucosa is continuously exposed to a variety of environmental signals. The gut immune system is challenged to balance homeostasis and distinguish commensal microorganisms from pathogenic bacteria, which is essential for eliminating pathogens and maintaining tolerance towards the microbiota and dietary antigens (15). The immune system associated with the intestinal mucosal is mainly located in the GALT (21). The latter is organized in the diffuse lymphoid tissue of the intestinal lamina propria, the intraepithelial lymphocyte compartment, and organized lymphoid follicles, such as Peyer's Patches (15,21). Some examples of immune cells that are involved in host protection within the intestinal lamina propria include monocytes, macrophages, neutrophils, dendritic cells (DCs), T and B lymphocytes, mast cells, and eosinophils **(Figure 1)** (15).

Microorganisms can be recognized by intra- and extracellular pattern-recognition receptors (PRRs) of immune cells and epithelial cells, which detect the pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs) from microorganisms. In turn, PAMPs and MAMPs are essential and well-preserved microorganism components that microbes cannot modify easily and are absent from the host (22–24). Among PRRs, detecting signals through cell membrane-bound Toll-like receptors (TLR) by mucosal tissue-resident cells is the first step of a protective host response (22). The engagement of TLRs with microbial PAMPs induces the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ)

(22). These events lead to cell activation and trigger the release of cytokines, such as Interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, and surface costimulatory molecules for antigen presentation in tissue-resident antigen-present cells (APCs), such as macrophage and dendritic cells (22,25). Moreover, the release of chemokines and cytokines during infections of the gut mucosa prompts the migration of immune cells like monocytes and neutrophils from the peripheral blood to the site of infection (26). Neutrophils have a particularly critical role in protecting from and controlling infections and will be discussed in detail later in this literature review.

The pro-inflammatory environment during infections induces the maturation and migration of APCs, especially dendritic cells, to mesenteric lymph nodes through afferent lymphatic vessels, thereby regulating the adaptative immune responses (27). The priming of naïve T-lymphocytes through APCs leads to the differentiation and clonal expansion of effector T helper (Th, identified by CD4+ expression) cells and the subsequent activation of B cells for antibody assembly (27). The CD4+ effector T cells exit the mesenteric lymph node and migrate to the site of infection. In the gut, CD4+ T cells are essential in coordinating an adequate response for pathogen control and clearance (28).

T cells are classically subdivided according to the pattern of cytokines produced upon activation (15). Lamina propria CD4+ T helper cells are essential for resolving a variety of infections. Among T helper cells, Th1 cells mainly produce IL-2 and interferon (IFN)-γ and are usually involved in responses against intracellular pathogens (29). Th2 cells predominantly produce IL-4, IL-5, and IL-13 and are critical for response against helminths (15,29). Th17 cells typically produce IL-17 and IL-22 cytokines, and their activation mainly occurs upon exposure to IL-23 cytokine release by APC. Th17 cell

responses play essential roles in protection against bacterial and fungal infections (15,30).

Among the various cytokines produced in the gut, the interleukin IL-22 is a cytokine that plays a crucial role in gut protection. It is released mainly by Type 3 innate lymphoid cells (ILC3s) and T CD4+ cells upon stimulation of proinflammatory signals, such as IL-23, produced by myeloid cells (31). ILC3s are part of the innate immune response that is mainly described as resident-tissue cells in peripheral tissues that are able to respond promptly via the production of cytokines, such as IL-22, due to their location and effector phenotype that does not require priming to expand and activate (32–34). Upon infection, ILC3s are an important early source of IL-22 (33). On the other hand, Th22 and Th17 are between the main populations of the adaptative response responsible for producing and maintaining the levels of IL-22 upon a tissue perturbation by infection and/or inflammation (35–37). In the gut, the cytokine IL-22 is critically involved in different aspects of the mucosal defense, ranging from epithelial barrier homeostasis to the production of mucus and antimicrobial peptides to control and resolve inflammation (38).

During infection, the production of IL-22 stimulates the release of antimicrobial proteins by epithelial and immune cells, such as lipocalin-2, calprotectin, and regenerating islet-derived protein 3-beta and -gamma (Reg3β and Reg3γ) (38–42). Lipocalin-2 binds to bacterial siderophores, which are small molecules essential for bacteria to scavenge iron (43,44). Calprotectin chelates metals, including manganese and zinc (45). In turn, the restriction of metal acquisition by these proteins is a crucial mechanism for controlling the expansion of pathogens during infection (43). Also, Reg3 proteins exerts direct antibacterial activity by binding to components of bacterial membranes such as peptidoglycan (46) and lipid A (47).

Although protection against hazards is essential for the host to survive, immune cells are challenged in distinguishing between self and potential threats to the host once a directed response against the body's antigens can be detrimental. The gut immune tolerance within the gastrointestinal (GI) tract prevents the body from initiating harmful or exaggerated immune responses against antigens found in the gut, such as food particles and commensal bacteria (48,49). Some examples of key mechanisms of gut immune tolerance include the actions of regulatory T cells (T regs), immune cells in the GALT, the mucosal barrier, and the gut microbiota (48). T regs can induce tolerance by expressing immune suppressive cytokines such as TGF−β and IL-10 (50–52) or by limiting the proliferation of cells (51,53,54). Some types of GALT-associated DCs, for example, have been shown to present tolerogenic and immunoregulatory responses, thus inducing T regs cells (55–57). The physical epithelial barrier, in addition to the production of mucus and antimicrobial proteins, reduces the excessive contact between immune cells and antigens (58). Finally, the microbiota, through different mechanisms, helps to train the immune response (49,56). Together, these mechanisms educate the immune system to distinguish between harmful pathogens and benign antigens, promoting a balanced and tolerant immune response within the gut, which is crucial for maintaining gut health and preventing unnecessary inflammation and autoimmune reactions within the digestive system (56).

Although the microbiota is not part of the mucosal immune system per se, it is the first barrier pathogens encounter within the host. The microorganisms inhabiting the host prevent pathogens from gaining access to nutrients, a phenomenon termed “colonization resistance” (3,59). This is well supported by studies in germ-free mice and antibiotic-treated mice, which develop more severe infections with enteric pathogens than mice with

a normal microbiota (60–62). The microbiota also prevents the overgrowth of pathogens through signals that stimulate host immunity to induce the expression of antimicrobial peptides by Paneth cells and epithelial cells (9).

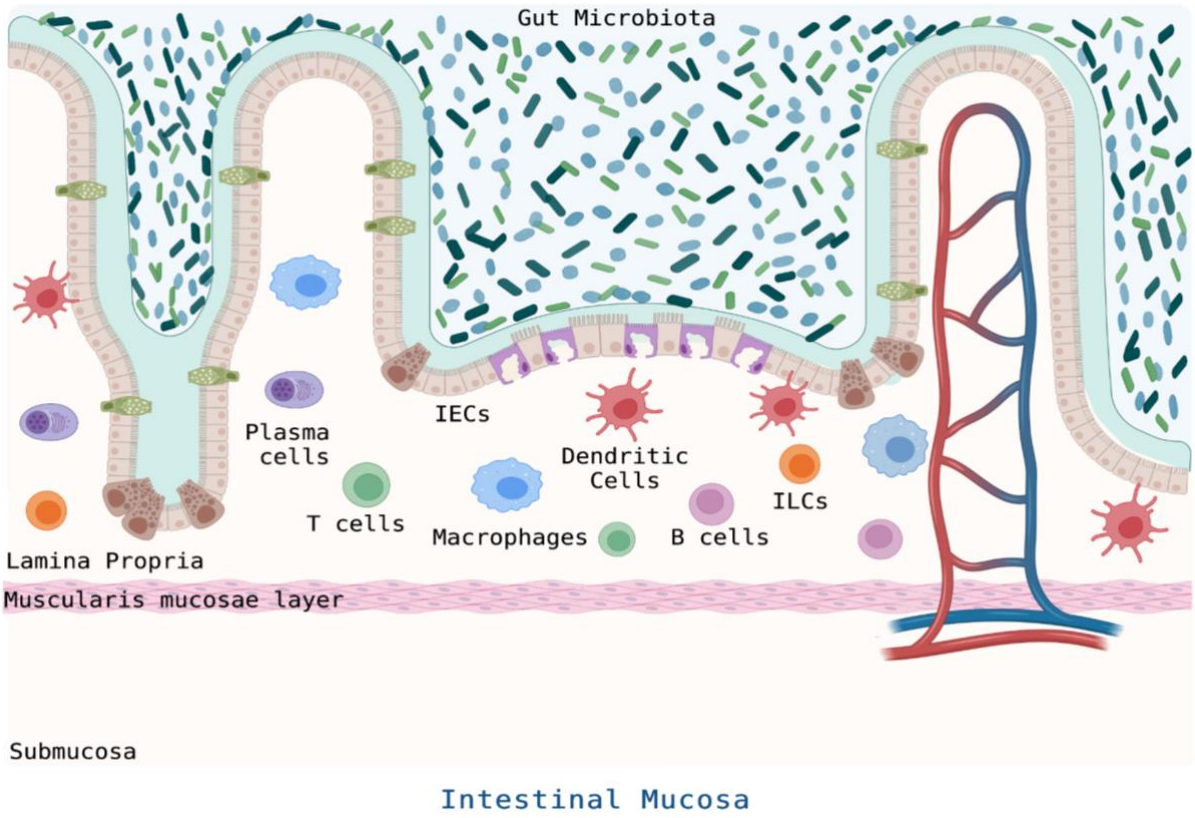

Figure 1. General Schematic of the intestinal mucosa. The gut is a muscular tube consisting of the luminal epithelium, underlying lamina propria, and muscularis mucosae layers. The luminal epithelium consists of a single layer of Intestinal epithelial cells (IECs) and specialized cell types such as goblet cells, enterocytes, enteroendocrine cells, Microfold cells, and Paneth cells. The lamina propria is a connective tissue layer with various immune cells such as B cells, dendritic cells, innate lymphoid cells (ILCs), T cells, plasma cells, and macrophages, among others. This layer separates the epithelium from the muscularis mucosae, and this organization is essential for the gut's physiological functions (Figure created with Biorender).

1.3. Neutrophils and host protection

Neutrophils are the most abundant phagocytic cell type of the innate immune system and constitute the first line of defense against infection. Neutrophils are derived from self-renewing hematopoietic stem cells in the bone marrow, which is regulated by

the cytokine granulocyte monocyte colony-stimulating factor (GM-CSF) (63). Mature neutrophils are retained in the bone marrow by expression of surface C-X-C Motif Chemokine Receptor 4 (CXCR4) and high constitutive production of its ligand C-X-C Motif Chemokine Ligand 12 (CXCL12) from osteoblast cells (64,65). Mature neutrophils are constantly released into the peripheral blood, which is greatly enhanced upon inflammatory stimuli or infection, resulting in more rapid mobilization of circulating neutrophils (66). The granulocyte colony-stimulating factor (G-CSF) promotes neutrophil exit from bone marrow by interfering with CXCR4-CXCL12 interaction (67). Besides, the chemokine receptor CXCR2 facilitates the egress of neutrophils from the bone marrow due to the constitutive production of its ligands CXCL1 and CXCL2 by endothelial cells (68).

During inflammation, tissue-resident cells such as macrophages and mast cells release chemoattractants and proinflammatory mediators (e.g., CXCL1, IL-1β, and TNF), which in turn facilitate the recruitment of neutrophils from the peripheral blood into the inflamed tissue (65). Adherence to the endothelium is initially mediated by a loose interaction between the neutrophil P-selectin glycoprotein ligand 1 (PSGL-1) and the endothelial E and P-selectins. Next, a firm adhesion is mainly mediated by the high affinity interaction of the integrin LAF-1 (leukocyte function-associated antigen 1) on neutrophils with ICAM1 (intracellular adhesion receptor 1) on endothelial cells (65,67). Binding promotes the vascular adhesion of neutrophils and, consequently, their translocation into inflamed tissue (65). In addition, increased expression of the CXCR2 ligands, CXCL1 and CXCL2, is involved in guiding neutrophils to the injured tissue (69).

Neutrophils are essential in controlling infection due to their high quantity of intracellularly stored granules that contain various proinflammatory and antimicrobial

proteins (70). At the site of infection, recognition of pathogens through NOD-like receptors (NLRs) and TRLs on the surface of neutrophils induce responses that include intracellular activation, degranulation, phagocytosis, and release of neutrophil extracellular traps (NETs) **(Figure 2)** (71). After phagocytosis, neutrophils eliminate pathogens via the production of reactive oxygen species (ROS) in the phagosomes and by producing and releasing antimicrobial peptides such as myeloperoxidase, elastase, cathepsins, defensins, lactoferrin, and lysozyme (70,71).

Another mechanism of neutrophils that contributes to immune protection is the release of neutrophil extracellular traps (NETs) (72) in a process known as NETosis. NETs can be formed in two ways; one way is driven by the cell death pathway that begins with nuclear delobulation and dismantlement of the nuclear envelope until complete abrogation of cellular polarization, followed by chromatin decondensation and plasma membrane rupture. The alternative route is known as non-lytic NETosis, which occurs independently of cell death and is related to the secreted expulsion of nuclear chromatin followed by the release of granule protein (71). Although an excessive release of NETs can be detrimental to the host's health, studies have shown that NETs have an essential role in the host's defense against pathogenic microorganisms (71,73–75).

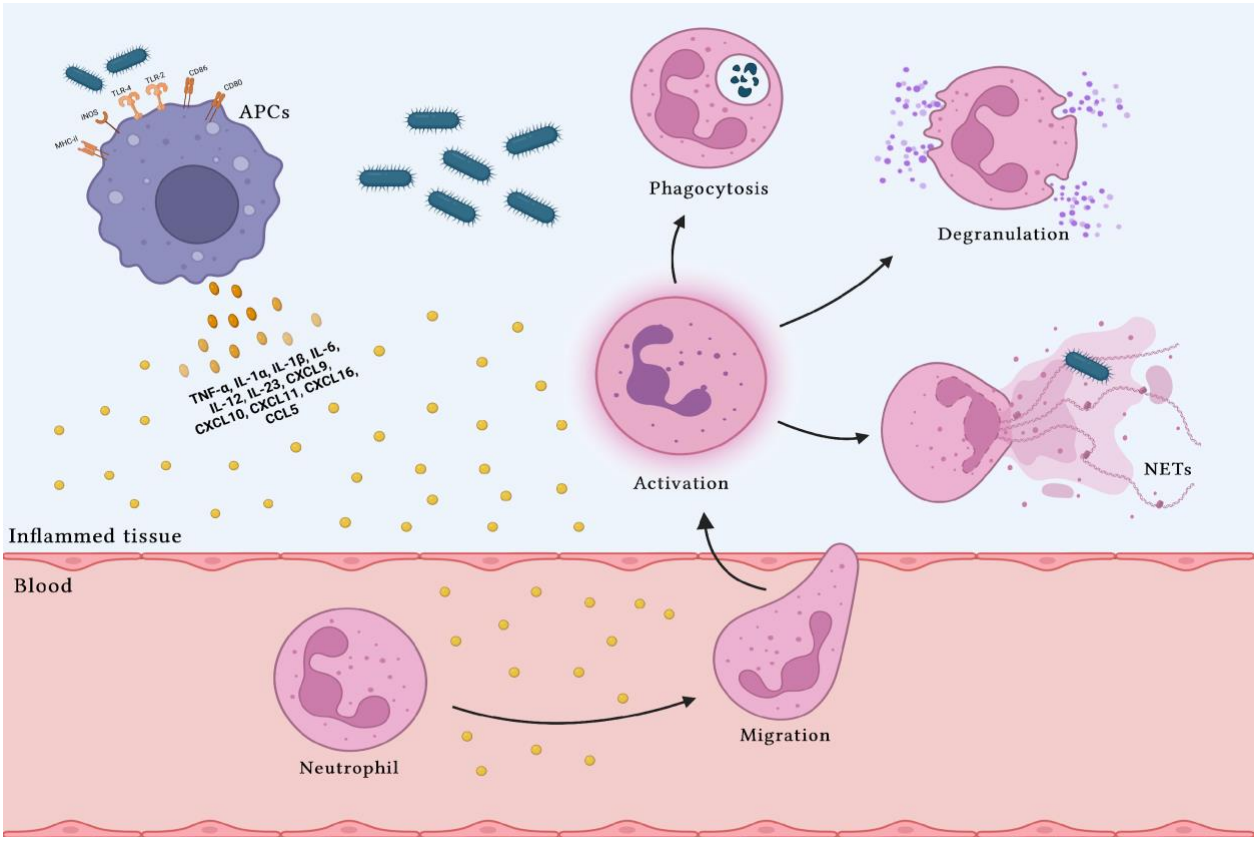

Figure 2. Neutrophils mediate responses at the site of infection. The figure elucidates the intricate process of neutrophil response to a stimulus triggered by a microorganism. Upon the production of cytokines and chemokines by tissue-resident antigen-presenting cells, neutrophils migrate from the blood to the injured tissue, where they become activated by the local environment. Upon activation, neutrophils engage in pathogen elimination through phagocytosis, respiratory burst, degranulation, and NETosis (Figure created with Biorender).

1.4. Pathogenic *Escherichia coli*: EHEC and EPEC

The human gut microbiome is a complex community composed of 10^{13}-10^{14} microorganisms and forms a mutualistic relationship with their host (3,76). Among the various genera that compose the human microbiota, the genus *Escherichia* is a widely distributed group inhabiting the large intestine of humans and other warm-blooded species (77,78). The Gram-negative bacterium *Escherichia coli* (*E. coli*) belongs to the *Escherichia* genus and represents a harmless commensal that frequently colonizes the human gut and rarely causes disease. However, throughout evolution, some clones have

acquired virulence genes that can cause severe illness in healthy individuals, therefore being designated pathogenic *Escherichia coli* (79).

Due to the genetic diversity of pathogenic *E. coli*, diseases caused by this microorganism are manifold. They can range from gastrointestinal tract infections, mainly assigned to enteric pathogenic *E. coli*, to extraintestinal infections involving sites such as the urinary tract, bloodstream, and central nervous system (80). Enteric pathogenic *E. coli* is usually restricted to the gastrointestinal tract and is classified according to the primary mechanism of virulence. So far, seven categories of enteric pathogens have been described: enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), adherent-invasive *E. coli* (AIEC), diffusely adherent *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (79,81,82).

The diarrheagenic EHEC and EPEC are human pathogens transmitted by the fecal-oral route and are primarily associated with consuming contaminated food and water (81). EHEC is a subset of Shiga toxin-producing *E. coli* (STEC), well-known for causing hemorrhagic colitis, followed by the potential development of Hemolytic Uremic Syndrome (HUS) in healthy individuals (79). HUS is characterized by the triad of thrombocytopenia, mechanical hemolytic anemia, and acute kidney injury (83) and more often affects susceptible patient groups, including infants, children, and the elderly (83,84). EHEC outbreaks have a high prevalence in developed countries, although developing countries such as Argentina have presented the highest HUS incidence in children under the age of five (80). Antibiotic treatment is not recommended for STECs due to the enhancement of Shiga-toxin release and increased development of UHS (85). Treatment is supportive and includes rehydration and, in some cases, dialysis (86).

Diarrhea caused by EPEC has been associated with severe disease and mortality in children worldwide. Although EPEC infections in developed countries have largely disappeared, they remain a significant public health concern in undeveloped countries (80) mainly for causing persistent and potentially fatal diarrhea in children under two years old (82,87).

Due to the histopathological lesions on intestinal epithelial cells, known as *Attaching* and *Effacement* lesions (A/E lesions), EHEC and EPEC are known as *Attaching and Effacing* pathogens (A/E pathogens). The ability to induce A/E lesions is conferred by a group of virulence genes denominated Locus of Enterocyte Effacement Island (LEE Island) (88). The LEE Island is a 35.6Kb pathogenicity island in their genome consisting of 41 genes organized into five operons called LEE1, LEE2, LEE3, LEE4, and LEE5 (88–90). Once activated, the LEE genes encode structural components of the type III secretion system (T3SS), regulators, chaperones, and effector proteins (90). Through the T3SS system, which resembles a needle-like structure, A/E pathogens inject effector proteins and the receptor Tir into the host cell (91). Tir binds to intimin, an adhesin in the bacterial cell membrane. Tir and effector protein insertion triggers host cell actin polymerization that leads to an accumulation of actin underneath the attached bacteria, leading to pedestal-like structures that promote the intimate attachment of A/E pathogens to the enterocyte membrane (92,93) **(Figure 3)**. The LEE island, as well as the A/E lesion, are not exclusive of EPEC and EHEC since bacteria such as the mouse pathogen *Citrobacter rodentium*, dog-enteropathogenic *E. coli*, porcine-enteropathogenic *E.coli* and rabbit EPEC (REPEC) also possess the LEE island and share similar characteristics of virulence (79,80,89).

New therapies and strategies have been studied to manage EPEC and EHEC infections. Nevertheless, since these bacteria are poorly pathogenic in mice, there is a limitation for *in vivo* studies in small laboratory models. In this context, *C. rodentium* constitutes an attractive alternative model for understanding the primary mechanisms used by A/E pathogens *in vivo* (94).

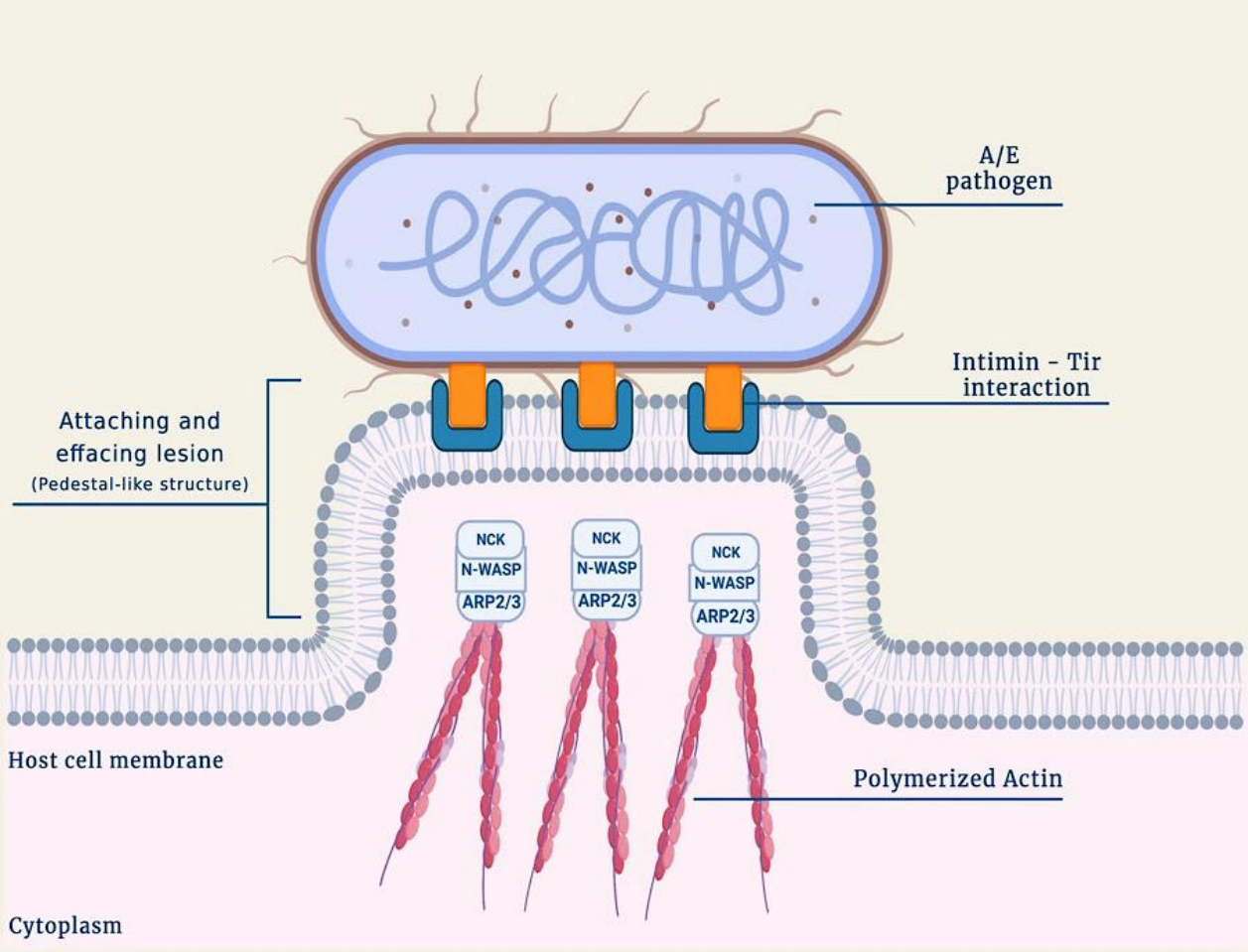

Figure 3. The pedestal-like structures are formed by Attaching and Effacing pathogens. The initial attachment of Attaching and effacing (A/E) pathogens is facilitated by surface adhesins. Subsequently, the receptor (Tir) is translocated within the host cells, binding to the bacterial surface protein intimin in the bacterial cells, promoting close attachment to host cells. The injection of effector proteins and the Tir-Intimin interaction leads to the recruitment of the Nck adaptor to a phosphorylated Tir residue, activating neural Wiskott-Aldrich syndrome protein (N-WASP). This activation triggers a significant pathway for actin polymerization, facilitated by the actin-related protein (Arp) 2/3 complex, culminating in the induction of a pedestal-like structure (Figure created with Biorender).

1.5. *Citrobacter rodentium*: an *in vivo* mouse-pathogen model

C. rodentium is a Gram-negative murine pathogen transmitted via the fecal-oral route that can cause colitis in laboratory mice. It was first described in the literature as atypical *Citrobacter freundii* after an outbreak in mouse colonies between the 1960s and 1970s (95). Later, it was found that *C. rodentium* was a mouse-restricted pathogen with 67% genetic similarity to EHEC and EPEC. The genome of *C. rodentium* also includes the LEE island, thus conferring the ability to form A/E lesions (89,96). Since *C. rodentium* is a natural mouse pathogen that is able to establish infection without antibiotic pretreatment, it has been the principal rodent model for studying diseases caused by A/E pathogens (97).

The susceptibility of inbred mice to *C. rodentium* depends on the genetic background as well as differences in the composition of the gut microbiota. Mouse strains such as C57BL/6 and Swiss Webster develop mild self-limiting disease with a typical infectious cascade, which can be divided into four phases: 1. The establishment phase, 2. the expansion phase, 3. the steady-state phase, and 4, the clearance phase (97). During phase 1, following inoculation and between 1 - 3 days post-infection (DPI), *C. rodentium* initially colonizes the lymphoid patches and adapts to the gut environment (98). In phase 2 (4-8 DPI), *C. rodentium* colonizes the intestinal epithelium cells by loose attachment mediated by fimbriae (94). Next, activation of LEE island genes results in intimate adhesion to the intestinal epithelial cells and initiates the formation of A/E lesions, a process that is mediated by environmental signals (91). The virulence of *C. rodentium* depends on the ability to perform A/E lesions, and studies have shown that the absence of Tir, intimin, and functional T3SS (all encoded by LEE island genes) renders *C. rodentium* to become avirulent (89,96,99).

During phase 3 (8-12 DPI), *C. rodentium* primarily occupies the distal part of the colon and cecum and shedding reaches a plateau at 10^8 and 10^9 colony-forming units (CFU) per gram of feces. Finally, in phase 4 (after 12 DPI), the host starts to clean the infection (97). In more susceptible mouse strains such as C3H and FVB, instead of recovery, the mice rapidly deteriorate after day 8 of infection and ultimately succumb to dehydration (97).

The microbiota is a crucial factor that dictates the host's susceptibility to infections. In *C. rodentium* infection, the microbiota affects the colonization efficiency and influences the outcome of the disease (100). Nevertheless, *C. rodentium* evolved mechanisms to overcome the colonization resistance promoted by the gut microbiota. *C. rodentium* is a facultative anaerobic bacterium capable of surviving in aerobic and anaerobic conditions. The oxygen gradient of the gut is not homogeneous. It varies depending on the spatial location, with higher concentrations at the submucosa and epithelium to near anoxia in the lumen (101). The anoxic environment is beneficial to obligate anaerobic bacteria such as *Clostridia*, which have an essential role in producing short-chain fatty acids such as butyrate (102). Butyrate, for instance, has immunomodulatory activity and is the primary energy source for colonic epithelial cells (103–105). The butyrate metabolism of colonic cells induces mitochondrial β-oxygenation through oxygen consumption, thereby limiting its diffusion and providing a low-oxygen environment at the luminal border (97,103,106). *C. rodentium* can disrupt epithelial mitochondria, forcing IECs to switch their regular metabolism to aerobic glycolysis to produce ATP. This results in lower oxygen consumption by IECs and greater oxygen diffusion into the lumen, thereby providing a fitness advantage for *C. rodentium* (97,106,107).

In mice with both severe and mild disease, *C. rodentium* infection leads to transmissible colonic crypt hyperplasia (TCCH), the hallmark of *C. rodentium* infection (94,95,97,108,109). TCCH is characterized by the induction of tissue damage repair responses due to the excessive proliferation of LGR5+ stem cells and partially differentiated transit-amplifying cells. This process leads to an accumulation of undifferentiated colonocytes at the luminal surface of the colon and loss of mature epithelial cells, such as enterocytes and goblet cells, with the result of a thickened colon mucosa (94,95,97,109). Also, it could facilitate epithelial oxygenation, which is advantageous for *C. rodentium* (110).

1.6. *C. rodentium* and the immune response

During infection, components of the T3SS, Lipopolysaccharide (LPS), an outer membrane portion constitute with a oligosaccharide core, Lipid A and O-antigen (111) or PAMPs of *C. rodentium* are recognized by immune cells, leading to the activation of the downstream adaptor molecule myeloid differentiation primary-response 88 (MYD88) (112). This leads to the activation of NF-kβ, which induces the production of proinflammatory cytokines, including IL-6, IL-12, IL-23, and tumor necrosis factor in myeloid cells (113). Pro-inflammatory responses induce various antimicrobial processes, including the recruitment of neutrophils, macrophages, dendritic cells, and the expression of inducible nitric oxide synthase (INOS) that is essential for controlling *C. rodentium* infection (94). However, the proinflammatory environment also amplifies the proliferation of epithelial cells during TCCH.

The chemokines induced in the colon upon infection promote the influx of neutrophils to the mucosa. In this context, CXCR2 receptor-mediated recruitment of neutrophils is essential for host defense and protection against *C.* rodentium (114). Besides, neutrophil antimicrobial responses such as NET formation ensure *C. rodentium* clearance and mucosal protection during infection (75).

The pro-inflammatory environment initiated by the innate immune system induces the differentiation and expansion of effector T cells such as Th1, Th17, and Th22 (115–117). The production of IL-17 induces expression of the cytokine IL-22 and further promotes the recruitment of neutrophils. Furthermore, ILC3s are an important source of NOD-dependent IL-22 induction (94). Studies have shown that IL-22 is essential for the resolution of *C. rodentium*-mediated disease since the absence of IL-22 leads to higher levels of epithelial damage, systemic bacterial burden, and mortality in mice (39,118). However, the underlying mechanisms of IL-22-mediated protection in the context of A/E pathogens infection remain unknown.

1.7. Chemical signaling in pathogenic *Enterobacteriaceae*

The successful establishment of infection depends on the ability of pathogens to sense the local environment and regulate virulence genes according to changes at the colonized niche. Bacteria can detect environmental fluctuations and establish cell-cell communication through the production and diffusion of small chemical molecules or signals recognized by receptors in their membrane (119). Changes such as increasing bacterial colony numbers, hormones produced by the host, and variations in the sugar concentration can be sensed by bacteria to modulate their response accordingly (79,120–122).

The inner membrane two-component system sensor QseC is a histidine kinase receptor that, together with its cognate regulator QseB, promotes gene modulation in Gram-negative bacteria. QseC senses mammalian adrenergic hormones, norepinephrine, epinephrine, and the autoinducer-3 secreted by bacteria (123,124). The binding of these molecules to the QseC receptor leads to the phosphorylation of QseB, which triggers a chemical signaling cascade that modulates gene expression in bacteria, specifically the upregulation of virulence genes in pathogens (125,126). Pathogens can have access to norepinephrine, and epinephrine is mainly synthesized at the end of postganglionic sympathetic nerve fibers and by chromaffin cells of the adrenal medulla reaching the gut via the bloodstream (17), which could be influenced by the microbial community inhabiting the gut and dietary sources (17,127,128). Besides, studies have shown that immune cells could also be another source of catecholamines (129).

Previous studies have shown that the QseC receptor is not essential for bacterial growth but is involved in pathogenic mechanisms. In *Salmonella enterica serovar* Typhimurium, blockage of the QseC sensor decreases *in vivo* colonization (124). In A/E pathogens, it has been demonstrated that QseC, directly and indirectly, regulates LEE gene expression (120,124,126,130). In EHEC, the blockage of the QseC sensor led to a decrease in the number of epithelial A/E lesions (124). *C. rodentium*-infected C3J mice, which usually succumb to infection, show significantly improved survival rates upon infection with a *qseC* mutant, suggesting that QseC impacts the virulence of *C. rodentium* (131).

Although it is well established that the QseC sensor aids in A/E pathogen colonization, little is known about the role of QseC signaling in these pathogens to evade and resist immune responses during disease establishment. Thus, the contribution of

adrenergic hormone detection via QseC in A/E pathogens during *in vivo* infections, particularly *C. rodentium*, needs further investigation.

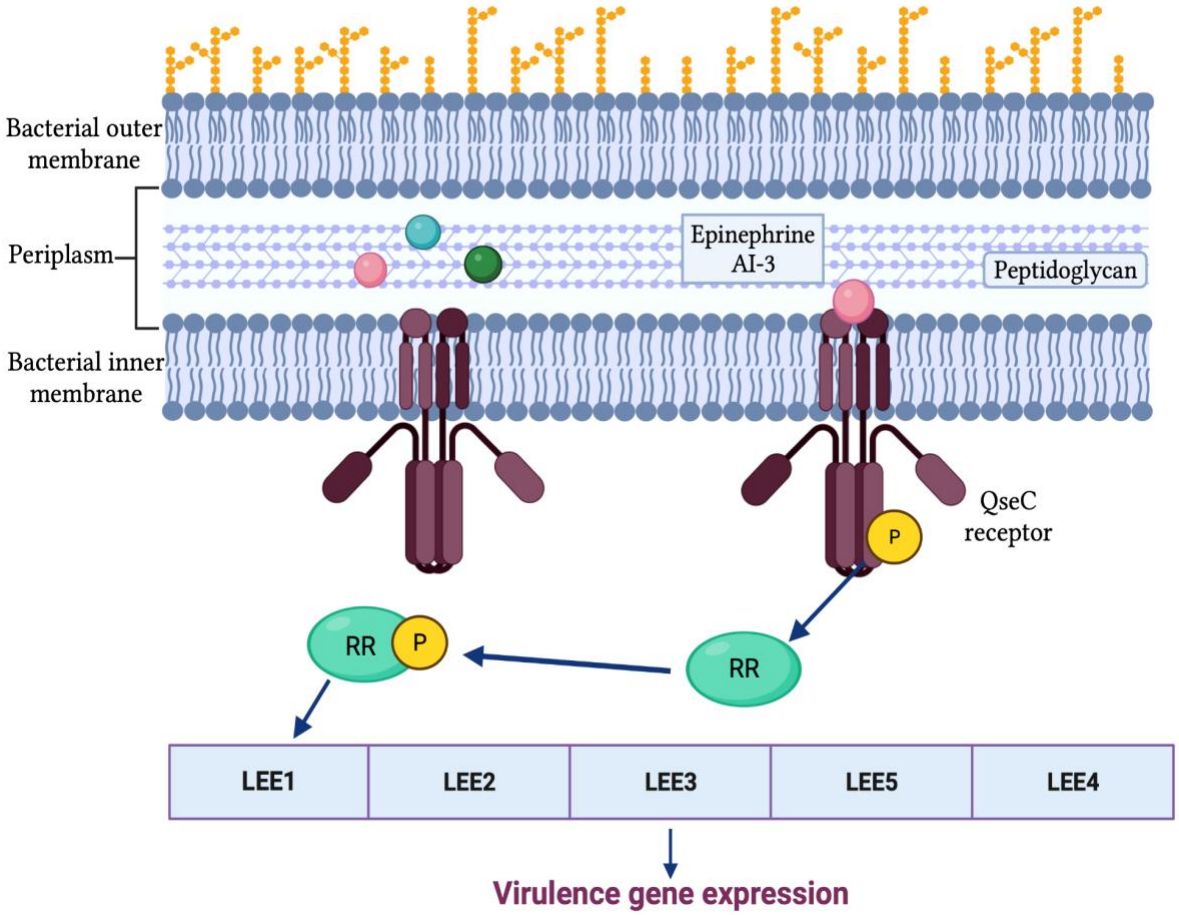

Figure 4. The QseC sensor regulation in A/E pathogens. The histidine kinase sensor QseC detects mammalian adrenergic hormones such as epinephrine and norepinephrine and the bacterial autoinducer-3 produced by bacteria, leading to the phosphorylation of Response Regulators (RR), such as QseB. The phosphorylated RR initiates a signaling cascade that leads to the activation of genes of the Locus of Enterocyte Effacement (LEE) pathogenicity island, thus modulating virulence gene expression in A/E pathogens (Figure created with Biorender).

Hypothesis and Objectives

Initiating effective immune responses is critical for the host to control and resolve infectious diseases. On the pathogen side, the activation of virulence genes is essential to disturb the microbiota-host homeostasis, induce inflammation, and evade antimicrobial response for successful colonization.

A/E pathogens use the QseC sensor to regulate their T3SS for colonization. Nevertheless, the contribution of the QseC sensor to the increase of virulence and inflammation induced by A/E pathogens *in vivo* is poorly understood. Only a few experiments have been conducted with the natural A/E mouse pathogen *C. rodentium* to understand the role of sensing adrenergic signals through the QseC sensor for evading and resisting the immune response to establish infection in mice. Thus, studies are necessary to comprehend the contribution of this sensor to host-pathogen interactions. On the host side, studies have shown that mice lacking the cytokine IL-22 succumb to *C. rodentium* infection, although the exact mechanism(s) remain elusive. IL-22 regulates various antimicrobial responses that protect the host from pathogens, but the downstream mechanisms of IL-22-mediated protection from *C. rodentium* infection need to be better understood. In this way, we hypothesized that the QseC sensor participates in *C. rodentium* resistance to the host immune response, thus contributing to the establishment of *C. rodentium* infection, and the protection mediated by IL-22 might be dependent on one of its antimicrobial-regulated peptides during *C. rodentium* infection.

Overall, this work proposes to extend the knowledge of the disease mechanisms of the mouse pathogen *C. rodentium* as an important A/E model during *in vivo* infection. Specifically, our work sought to address two main aims:

1) Investigate the impact of chemical signaling through the QseC sensor for establishing *C. rodentium* infection and resistance to the host immune response.

2) Elucidate the mechanisms of mucosal immune protection conferred by IL-22 during *C. rodentium* infection for resolution of disease.

Chapter II: Adrenergic signaling via QseC sensor in *Citrobacter rodentium* and its role in modulating immune responses during infection

2.1. Introduction

Diarrhea caused by enteric pathogens is associated with severe disease and mortality worldwide. Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) are human pathogens transmitted by the fecal-oral route that can form *attaching and effacing* (A/E) lesions, which is facilitated by genes in the Locus of Enterocyte Effacement Island (LEE Island) (88). LEE-encoded genes induce the Type 3 Secretion System (T3SS) that injects effector proteins and the receptor Tir into the host cell, thereby promoting the intimate attachment of A/E pathogens to the enterocyte membrane (91). Since EPEC and EHEC are human pathogens, the related pathogen *C. rodentium* has been used in mice as a suitable alternative to understand the mechanisms underlying A/E pathogenicity (89,96).

The disease caused by A/E pathogens is characterized by intestinal inflammation and diarrhea. To establish infection, pathogens use receptors in their membrane to sense environmental changes and respond appropriately. The QseC sensor responds to mammalian adrenergic hormones, norepinephrine, and epinephrine (123,124). In *C. rodentium*, EHEC, and EPEC, binding of these molecules to the QseC receptor triggers the phosphorylation of QseB for the expression of virulence genes via the direct and indirect regulation of LEE genes (120,124,126,130).

The breach of the intestinal barrier by *C. rodentium* induces inflammatory responses that are mediated by the innate and adaptative arm of immunities. Studies have shown

the QseC sensor is required for A/E pathogens to colonize mice effectively and to attach to epithelial cells *in vitro* (124,131). However, the contribution of the QseC sensor to gut inflammation induced by A/E pathogens *in vivo* is poorly understood. Here, we aimed to evaluate the impact of chemical signaling through QseC for resistance of *C. rodentium* to the host immune response and establishment of infection by using the self-limiting disease model in C57BL/6 mice.

2.2. Material and Methods

Bacterial strains: Bacterial strains used in the study were wild-type *Citrobacter rodentium* DBS100 (WT) and its isogenic mutant strain Δ*qseC C. rodentium* DBS100 (131) obtained from Prof. Vanessa Sperandio (University of Wisconsin, Madison), *C. rodentium* complemented strain (Δ*qseC/qseC*+), *Salmonella enterica* serovar Typhimurium WT (132), Δ*qseC* (124) and Δ*qseC/qseC*+ (133). Bacteria were cultured overnight aerobically in Miller Luria-Bertani (LB; 10 g/L NaCl) broth containing nalidixic acid (50 mg/mL for *C. rodentium*) or Streptomycin (100g/L for *S.* Typhimurium) at 37^{o}C with shaking. The next day, the bacterial cultures were washed, centrifuged, diluted, and resuspended in phosphate-buffered saline 1X (PBS, Gibco). The concentration of bacteria was adjusted according to the respective experiments.

Infection experiments: All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees from the University of California San Diego. Five-week-old female C57BL/6 mice from Jackson Laboratory were gavaged with 5x10^{9} WT or Δ*qseC* strains in 100 μL of PBS 1X. Before the infection, fecal samples were tested on MacConkey for *Enterobacteriaceae* negative control. Fecal samples were

collected on days 1, 2, 4, and 7 post-infection, weighed and homogenized in 1X PBS, serially diluted, and plated on LB agar containing Nalidixic Acid (NAL) for CFU enumeration. After 7 post-infection, mice were euthanized by CO_2 asphyxiation, and colons and spleens were collected. The spleen was homogenized in 5 mL of 1X PBS, serially diluted, and plated on LB-NAL for bacterial counts (CFUs). Colons were collected, assessed for length, and were either used for flow cytometry experiments or preserved for subsequent gene expression and histopathology.

Neutrophil *in-vivo* depletion experiments: Five-week-old female C57BL/6 mice from Jackson Laboratory either received 200 μL of anti-CXCR2 antiserum (raised in rabbits, Mehrad et al. 1999) or 250 μg of a monoclonal anti-Ly6G antibody (clone 1A8, Biolegend) intraperitoneally one day before infection and on day 3 post-infection. Control animals received 200 μL of rabbit gamma globulin for anti-CXC2 or rat gamma globulin for anti-Ly6G experiments (Jackson ImmunoResearch).

Macrophage phagocytosis assays: Bone marrow-derived macrophages were obtained from C57BL/6 WT mice. Briefly, mice were euthanized by CO_2 asphyxiation, and femurs and tibias were collected. Next, epiphyses were cut with a scalpel and bone marrow was flushed out with sterile RPMI (Gibco), spun down at 300xg, lysed with 1X Red Cell Lysis Buffer (in-house), washed with 1X PBS, and resuspended in RPMI medium complemented with fetal bovine serum (FBS, Life Technologies) 10%, 100x Antibiotic/Antimycotic (Gibco), and 30% of supernatant from L929 cells (containing macrophage colony-stimulating factor). After 7 days of macrophage differentiation and 24 hours before infection, cells were detached using iced cold-PBS 1X, collected, washed

with PBS, and re-seeded into a 24-well plate at a density of $1x10^6$ cells/mL in RPMI/ 10% FBS. The next day, overnight cultures of *C. rodentium* were opsonized with 20% normal mouse serum (collected from in-house WT animals) for 30 minutes at 37ºC. After opsonization, the bacterial strains were added to the macrophage cultures at a multiplicity of infection (MOI) = 10 and incubated for 30 minutes at 37ºC. For the enumeration of phagocytosed bacteria, macrophages were washed 3 times with PBS to remove extracellular bacteria, followed by cell lysis with PBS + 1% Triton-X (Millipore Sigma) and serial dilution for CFU enumeration. For quantification of intracellular survival of bacteria, complete media was replaced with RPMI + 10% FBS + gentamycin 100 µg/mL for 30 minutes. Next, the macrophages were washed once with 1x PBS and incubated in RPMI + 10% FBS + gentamycin 30 µg/mL for another 3, 6, or 9 hours at 37ºC. Subsequently, cells were lysed, and CFUs enumerated as described above.

Neutrophil killing assay: Bone marrow was obtained from C57BL/6 wild-type, C57BL/6 *S100a9*$^{-/-}$ (in-house) or C57BL/6 129S-Cybb$^{tm1Din/J}$ (Jackson Laboratory) mice and used for neutrophil enrichment by utilizing the EasySep Mouse Neutrophil Enrichment Kit (STEMCELL) following the manufacturer's instructions. After enrichment, $1x10^6$ neutrophils were seeded into a 96-well round-bottom plate in RPMI/10% FBS. *C. rodentium* strains from overnight cultures were opsonized with 20% normal mouse serum for 30 minutes at 37 ºC. After opsonization, the bacteria were added to the neutrophils at an MOI =10. The plate was spun down to promote interaction between cells and bacteria. Cells were incubated for 150 minutes or 240 minutes at 37 ºC with 5% CO_2. For elastase quantification, supernatants were collected after 150 minutes of infection. For the DNAse-I treatment assay, the DNase-I (200 U/mL) was dissolved in sterile water. For the

aminoguanidine (AG) assay, 1 mM/mL of AG was weighed and diluted in 1X PBS. The same volume of water or PBS 1X was added to the control groups. For CFU enumeration, 1:2 dilutions were performed in PBS supplemented with 2% Triton X-100, followed by serial dilutions and plating on LB agar containing antibiotic. The percentage of bacterial survival was calculated by dividing the number of bacteria recovered in the presence of neutrophils by the number of bacteria recovered from wells without neutrophils, then multiplied by 100, as previously described by Perez-Lopez et al., 2022 (134).

H_2O_2 killing assay: *C. rodentium* strains from overnight cultures were opsonized or non-opsonized with 20% normal mouse serum for 30 minutes at 37 ^oC. After opsonization, the bacteria were added to the plate and incubated for 150 minutes with 1mM of H_2O_2 at 37 ^oC with 5% CO_2. After incubation, serial dilutions and plating on LB agar containing antibiotics were performed for CFU enumeration. The percentage of bacterial survival was calculated by dividing the number of non-opsonized bacteria by the number of opsonized bacteria recovered from wells, then multiplied by 100.

Elastase quantification by ELISA: Supernatants from infected neutrophils were collected and used for elastase measurement utilizing a mouse Neutrophils elastase/ELA2 DuoSet ELISA (R&D systems) Kit according to the manufacturer's instructions. Briefly, a 96-well plate was coated with 100 µL of rat anti-mouse ELA2 capture antibody (1 µg/mL) and incubated overnight at room temperature. The next day, the plate was washed three times with wash buffer (0.05% Tween 20 in PBS) and blocked with Reagent Diluent (1% BSA in PBS) for 1h. Subsequently, the plate was washed three times with wash buffers, followed by incubation with 100 µL of diluted cell culture

supernatants for 2 hours. Thereafter, wells were washed 3 times and incubated with 100 µL of biotinylated sheep anti-mouse ELA2 Detection Antibody (100 ng/mL) for 2 hours. The plate was washed 3 times and incubated for 20 minutes with 100 µL of Streptavidin-HRP, followed by another 3 washes. Substrate solution (SIGMA*FAST*™ OPD, Sigma) was added to the wells, and the plate was incubated for 20 minutes in the dark. The reaction was quenched with 50 µL of 2N hydrogen sulfate (H_2SO_4). Absorbance was read at an OD of 492 nm using a Biotek Epoch Microplate Spectrophotometer. The cutoffs for ELISA assays were determined for each assay according to a previously described method (135).

Reactive oxygen species (ROS) assay: Neutrophils were incubated in the presence of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, 25 mM) (Invitrogen) for 30 min at 37ºC at 5% of CO_2 as previously described (136). After incubation with H_2DCFDA, cells were infected with *C. rodentium* WT or Δ*qseC* at MOI 10 and incubated for 4h. Cellular ROS production was determined by flow cytometry using a BD FACSCanto II cytometer, and data were analyzed using the FlowJo v10 software, as previously described by Perez-Lopez 2022 (134).

Histology: At day 7 post-infection, whole colons from infected mice with *C. rodentium* WT or Δ*qseC* strains were collected into Swiss rolls, formalin-fixed, paraffin-embedded, and sectioned for histopathology. The following parameters were scored as follows. Mononuclear infiltration scores are 0 for absent (normal sparse lymphocytic infiltrate), 1 for mild (diffuse increase in lamina propria, usually with plasma cells), 2 for moderate (lamina propria increased with basal localization aggregates displacing crypts, distinguish

normal MALT), and 3 for severe (lamina propria with submucosal infiltration). Crypt hyperplasia scores are 0 for absence and 3 for the highest score (severe). Epithelial injury scores are 1 for mild (crypt dropout/crypt distortion or surface epithelial damage, no frank erosion or ulceration), 2 for moderate (focal ulceration), and 3 for severe (multifocal or extensive ulceration). Neutrophilic crypt abscesses scores are 0 for absent, 1 for mild (lamina propria only), 2 for moderate (lamina propria plus cryptitis and crypt abscesses), and 3 for severe (sheet-like or submucosal infiltrate).

Lamina propria mononuclear cell isolation: Neutrophils, antigen-presenting cells, and T cells were analyzed in the colons of C57BL/6 mice seven days post-infection, as described earlier. Lamina propria mononuclear cells (LPMNCs) were isolated as previously described with minor modifications (137). Briefly, colons were removed and flushed with cold 1x PBS, cut longitudinally, minced, and placed in Calcium/Magnesium-free HBSS + 10% FBS containing 1mM DTT and 2mM EDTA (Sigma Aldrich). Tissue fragments were rotated for 10 minutes at room temperature (300rpm), followed by adding 1mM DTT, and rotated for another 10 minutes. Next, the tissue fragments were vortexed for 4 minutes at full speed, supernatants discarded, and tissue resuspended in HBSS (Corning) + 10% FBS. The suspensions were vortexed for another 2 minutes and resuspended in IMDM medium (Life Technology) supplemented with 20% FBS, 128U/ml type IV Collagenase, and 10U/ml DNAse II (both Sigma Aldrich), and digested for 60 min at 37°C. Subsequently, the tissue fragments were consecutively filtered through 100/70/40 cell strainers, spun down at 350 x g for 10 minutes, and resuspended in PBS.

Flow Cytometry: Lamina propria cells were isolated as described above, and approximately $1x10^6$ cells of each sample were transferred into respective wells of a round bottom 96-well plate. The plate was spun down at 350 x g for 10 minutes, supernatants were discarded, and cells were resuspended in PBS containing TruStain FcX to reduce non-specific binding and Zombie Aqua fixable viability dye (both BioLegend). The plate was incubated for 7 minutes at 4°C in the dark, followed by quenching with PBS/0.5% bovine serum albumin (flow buffer, FB). The plate was washed 3 times by spinning down at 350 x g for 10 min, and cells were resuspended in FB. Surface staining was performed using the following monoclonal antibodies at predetermined optimal concentrations: anti-mouse CD45-APC/Cy7 (clone 30F-11, BioLegend), anti-mouse CD3-PE (clone 145-2C11, eBioscience), anti-mouse CD11b-Pacific blue (clone M1/70, BioLegend), anti-mouse CD11c-FITC (clone N418, eBioscience), anti-mouse F4/80-PercP/Cy5.5 (clone BM8, BioLegend) and anti-mouse Ly6G-APC (clone 1A8, BioLegend). Cells were stained for 30 minutes at 4°C in the dark, followed by 3 washes with FB. Next, the cells were fixed with Fixation Buffer (Biolegend) for 20 minutes at 4°C in the dark, followed by two washes with FB. Fixed Cells were run on a BD FACSCanto II, and data was analyzed using FlowJo v10 software.

LPS Analysis: $5x10^8$ CFUs of overnight LB cultured WT and ΔqseC strains were double-washed with 1x PBS, resuspended in 100 µl lysis buffer, incubated at 95°C for 10 minutes, then briefly spun for 10 seconds. Next, 1.25 µl of Proteinase K (Viagen) was added and incubated overnight at 55°C. Following this step, 27 µl of samples were mixture with 3 µl of 2-Mercaptoethanol (Bio-Rad) and 4x Laemmli (Bio-rad), loaded in SDS-poly-acrylamide gel electrophoresis, and ran at 25 mA for 2 hours in 1X NUPAGE MES SDS

running buffer (Invitrogen). Next, the staining was performed following the Pro-Q Emerald 300 staining kit manufacturer's instructions. Briefly, the gel was fixed with 100 mL of fixation solution for 45 minutes on a shaker, washed 2x with 100 mL of wash solution for 15 minutes, and incubated in 25 mL with oxidizing solution for 30 minutes on shaker. After oxidization, the gel was washed 3x with 100 mL of wash solution for 15 minutes and stained with Pro-Q Emerald stock solution in the staining buffer for 120 minutes. Finally, the gel was washed 2x with wash solution for 15 minutes and revealed in Azute Biosystem UV302 excitation.

Statistical Analysis:

Statistical analysis was performed with GraphPad Prism v9. The Shapiro-Wilk test was used for normality distribution. For normally distributed groups, p values were calculated by unpaired student's t-test and Mann-Whitney U for unpaired data that were not normally distributed. If more than 2 groups were compared, one-way and two-way analysis of variance (ANOVA) followed by a multiple-comparison test was used.

2.3. Results

Previous studies have shown that the QseC sensor is essential for the virulence of pathogens (124). In C3J mice, which usually succumb to *C. rodentium*, the absence of QseC led to a dose-dependent reduction of mouse mortality (131). To determine whether the lack of a QseC sensor affects *C. rodentium* infection and the course of the disease in a self-limiting mouse model, C57BL/6 WT mice were gavaged with *C. rodentium* WT and Δ*qseC* strains. Mice were weighed, and fecal samples were collected at D2, D4, and D7 days post-infection. At the end of experiments on D7, colon lengths were measured since

a reduction in colon length indicates inflammation. Also, *C. rodentium* dissemination was evaluated by CFU enumeration in the spleen.

As shown in Figure 5A, there were no significant differences in body weight between mice infected with WT or Δ*qseC C. rodentium*. However, mice infected with the WT strain showed significantly higher colonization levels on D2, D4, and D7 post-infection as compared to the Δ*qseC* strain (Figure 5B), suggesting that the QseC sensor might have a role in the colonization of *C. rodentium*. Furthermore, mice infected with the WT strain presented shorter colons than mice infected with the Δ*qseC* strain (Figure 5C).

Next, we examined whether the ability of *C. rodentium* to disseminate would be impaired by the absence of QseC by enumerating splenic CFU. Mice infected with the WT strain showed a high number of CFU in the spleen; however, no CFU were detected in mice infected with the Δ*qseC* strain (Figure 5D). Mice infected with the WT strain demonstrated splenomegaly, which was reflected by significantly higher spleen weights as compared to mice infected with Δ*qseC* (Figure 5E). Together, these data suggest that the QseC sensor enhances *C. rodentium* virulence *in vivo*.

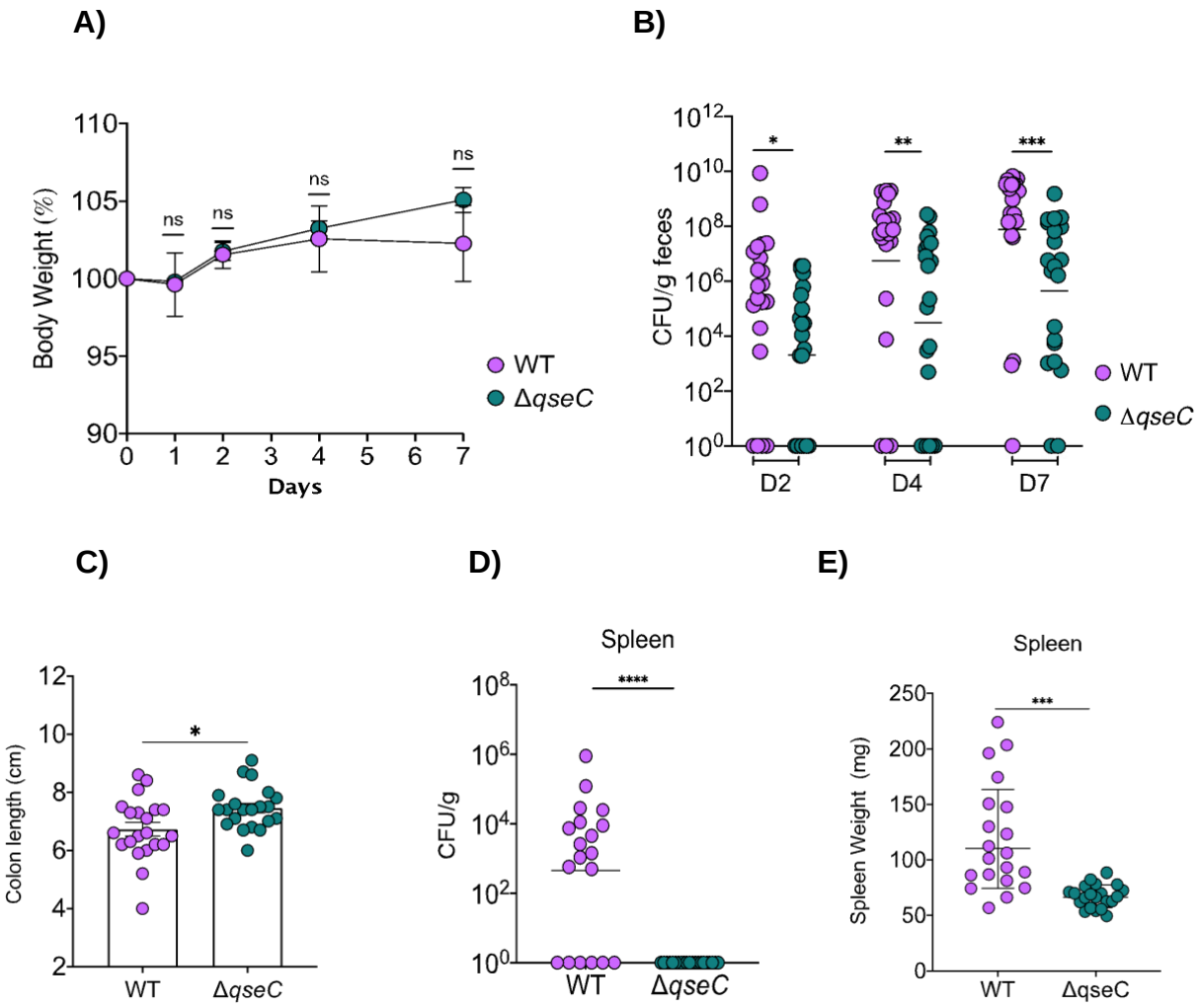

Figure 5. The QseC sensor is essential for *Citrobacter rodentium* virulence in C57BL/6 mice. **(A)** Weight course of mice infected with *C. rodentium* WT or *ΔqseC* at the indicated time point. **(B)** Analysis of CFU in fecal samples of mice infected with C*. rodentium* WT and *ΔqseC* on days (D) 2-, 4- and 7. **(C)** Colon were collected and measured at D7 post-infection. **(D)** CFU of *C. rodentium* in spleen at day 7 post-infection (D7). **(E)** Spleen weights from infected mice on D7. Bars represent the geometric mean ± SD. Each circle represents an individual mouse. Significant differences are indicated by p<0.05 (*), p<0.01 (**), p<0.01 (***), and p<0.0001 (****).

Swiss rolls from the colon were prepared for histopathological analysis. As shown in Figure 6A, colons from WT-infected mice displayed significantly higher pathology scores as compared to Δ*qseC*-infected mice (Figure 6A). The levels of mononuclear infiltrate, epithelial injury, and neutrophilic crypt abscesses were accentuated in mice infected with the WT strain (Figure 6C). Remarkably, the ability to induce crypt hyperplasia as a pathological hallmark of *C. rodentium* infection was decreased in mice

infected with the Δ*qseC* strain. Together, these data indicate that the signaling through QseC plays a role in *C. rodentium* virulence by eliciting inflammation and inducing crypt hyperplasia in enterocytes.

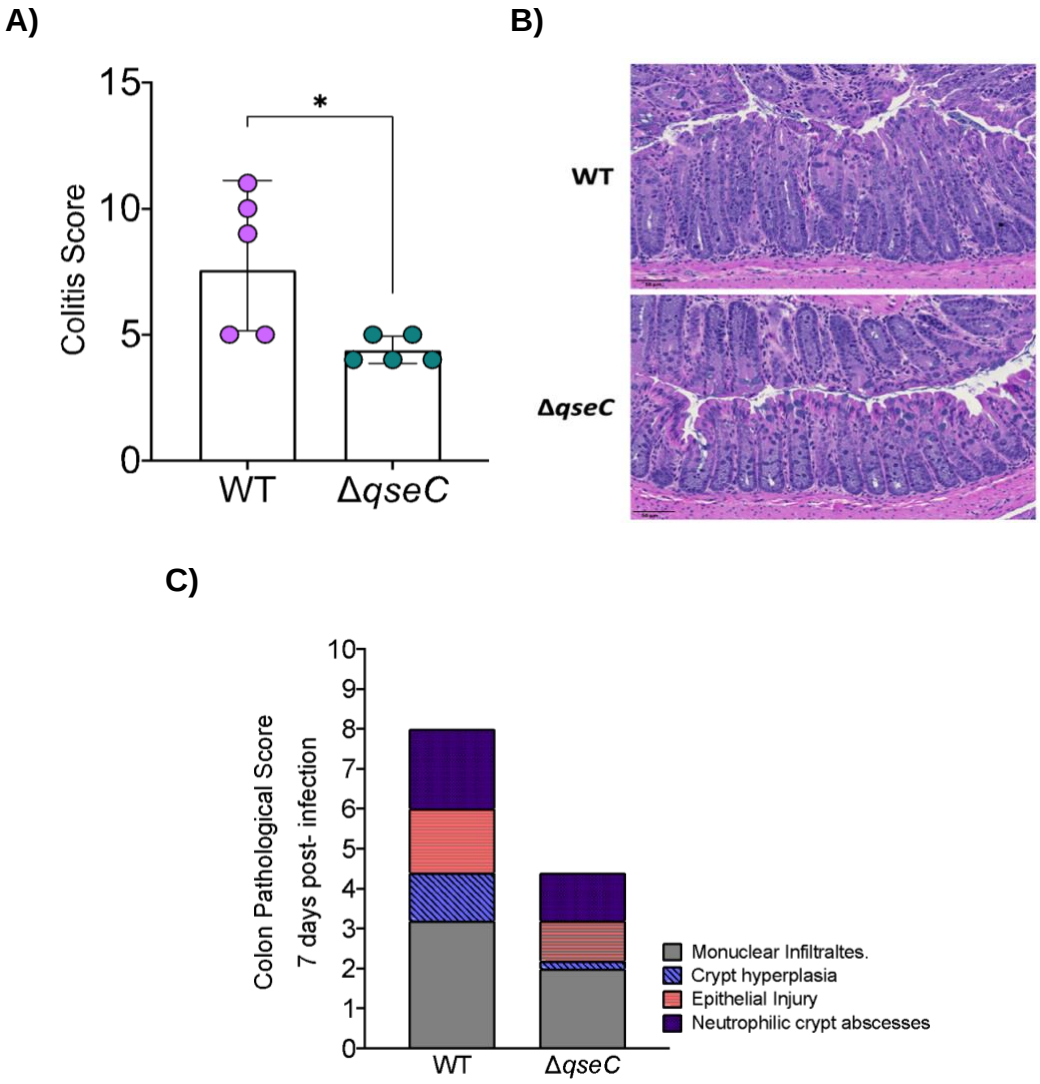

Figure 6. **QseC sensor exacerbates inflammation induced by *C. rodentium* in C57BL/6 mice. (A)** Colitis scores on day 7 post-infection**. (B)** Representative H&E stained colon sections from mice infected with WT or Δ*qseC C. rodentium*. **(C)** Histopathologic colon scores with subcategories on D7 post-C. *rodentium* infection. Each circle represents an individual mouse. Bars represent the geometric mean ± SD. Significant differences are indicated by $p<0.05$ (*).

Various gut immune cells are responsible for host protection during infections. As shown above, the histopathology data demonstrated that mice infected with the Δ*qseC* strain have significantly less inflamed colons than those infected with the WT strain. Next,

we characterized the immune cell infiltration of the colons following infection with WT and Δ*qseC* strains by flow cytometry on D7. The gating strategy is shown in Figure 7. Cells positive for CD11b+ and Ly6G+ were designated as neutrophils, CD11b-CD3+ as T cells, and antigen-presenting cells (APCs) were characterized by CD11b+ F4/80+ or CD11b+CD11c+.

The number of intestinal APCs CD11b+ F4/80+ was comparable in mice infected with the WT or Δ*qseC* strains (Figures 8A). However, mice infected with WT *C. rodentium* had increased numbers of infiltrating APCs CD11b+CD11c+ and neutrophils in the colon as compared to mice infected with the Δ*qseC* strain (Figures 8B and C). On the contrary, T cells were increased in mice infected with the Δ*qseC* strain (Figure 8D). These data suggest that the Δ*qseC* strain triggers the infiltration of immune cells as APCs CD11b+ F4/80+ comparable level to the WT strain. However, the lower inflammatory scores demonstrated in the histopathology during *C. rodentium* Δ*qseC* strain might be due to a decreased number of neutrophils and APCs CD11b+CD11c+ infiltrating the colon.

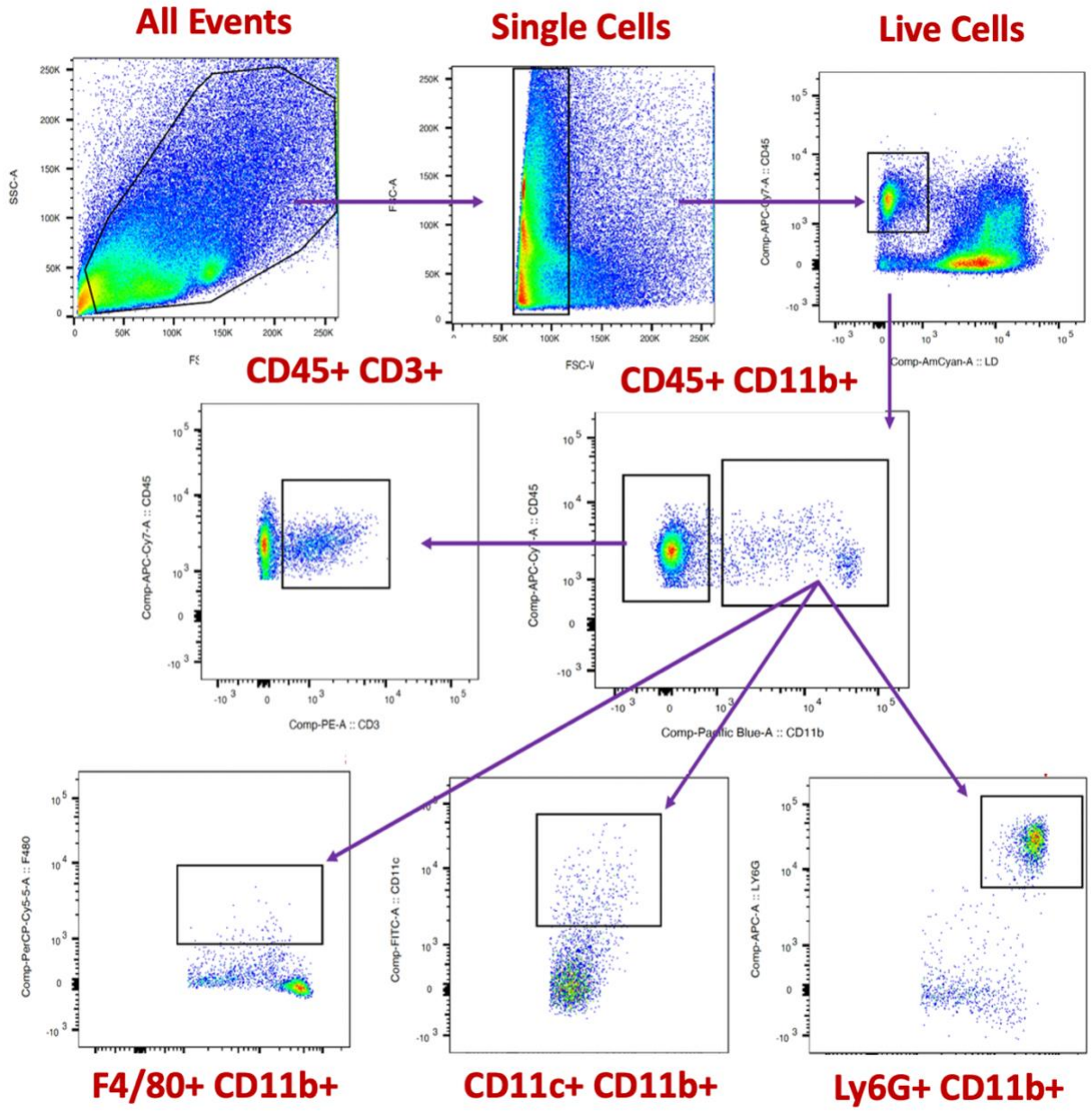

Figure 7. Gating strategy used in flow cytometry analysis for identifying colon immune cells. FSC-A and SSC-A gate was used for identifying lymphocyte populations. FSC-A and FSC-W were used for gating single cells and doublets exclusion. Single immune cells were gated based on CD45+ expression and Live and Dead staining for sorting live cells. The CD45+ and CD11b+ markers were selected for gating myeloid and non-myeloid cells. T cells, Macrophages, Dendritic Cells, and Neutrophils were gated based on their specific surface markers.

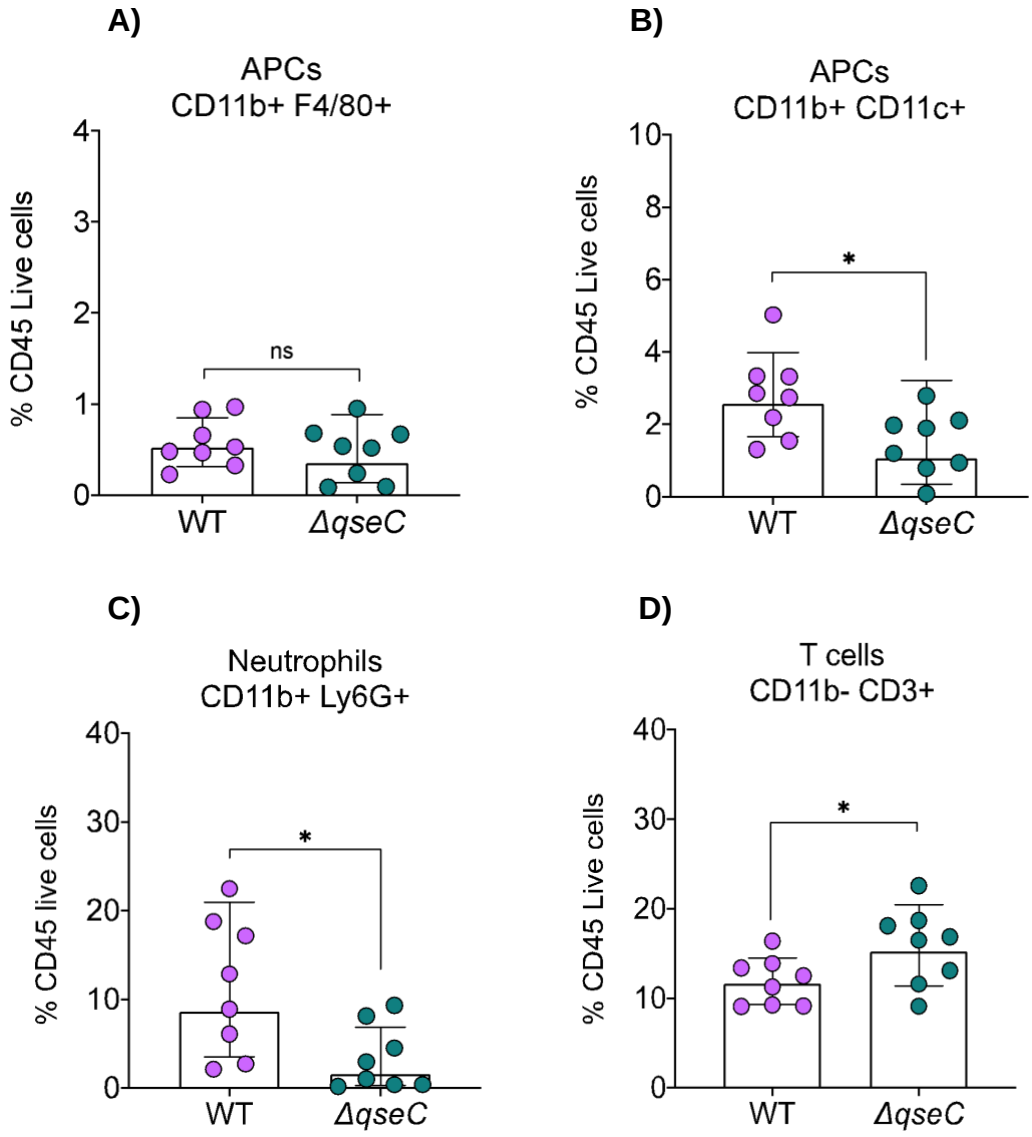

Figure 8. Analysis of immune cells isolated from the colon of C57BL/6 mice after infection with *Citrobacter rodentium* WT and Δ*qseC* strains. Immune cells isolated from the colon of infected mice after 7 days of *C. rodentium* infection. The frequency of neutrophils (CD45+ CD11b+Ly6G+), APCs (CD45+CD11b+F4/80+ and CD11b+CD11c+), and T cells (CD45+ CD11b-CD3+) from colon live immune cells were determined by flow cytometry. Each circle represents an individual mouse. Data compromised two independent experiments. Data represent geometric mean ± SD. Non-significant differences are indicated by ns; the significant differences are indicated by $p<0.05$ (*), $p<0.01$ (**), and $p<0.0001$ (****).

The infiltration of APCs CD11b+ F4/80+ in the colon of mice during infection was comparable between the Δ*qseC* strain and the WT strain, although colonization with *C. rodentium* Δ*qseC* was significantly decreased. Thus, we hypothesized that the QseC

sensor in *C. rodentium* could be involved in resistance to immune cell responses and that the Δ*qseC* strain could be more susceptible to immune cells.

Macrophages are essential innate immune cells in maintaining tissue homeostasis and eliminating pathogens during infection. These cells are part of the first line of defense against pathogens due to their tissue residency, ability to phagocyte microorganisms, and to produce bactericidal effectors such as nitric oxides and reactive oxygen intermediates (138).

To determine whether the presence or absence of the QseC sensor would affect the rate of *C. rodentium* phagocytosis and intracellular survival resistance in macrophages, bone marrow-derived macrophages were isolated and infected with *C. rodentium* WT and Δ*qseC* strains. For phagocytosis assays, macrophages were infected for 30 minutes until the infection was stopped by adding triton 1X. In intracellular survival experiments, gentamicin was added at a high concentration (100 µg/mL) after 30 minutes of infection to kill extracellular bacteria that were not phagocytosed, followed by incubation for 3, 6 or 9 hours with low gentamicin concentration (30 µg/mL). As shown in Figures 9A, 9B, 9C, and 9D, there were no differences in the phagocytosis or intracellular survival between the WT and Δ*qseC* strains in none of the time-point tested. Our result suggests that the QseC sensor is not essential for *C. rodentium* evasion of macrophage responses.

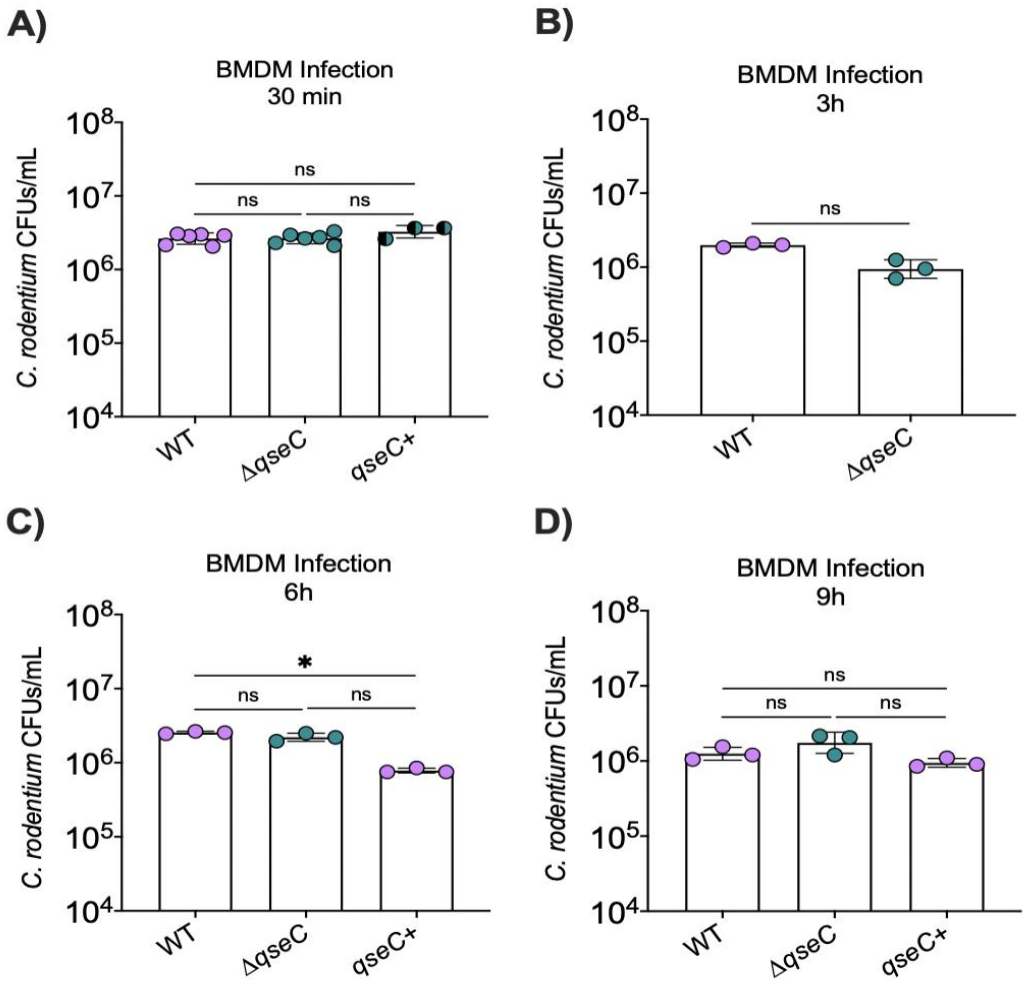

Figure 9. *Citrobacter rodentium* does not depend on QseC to evade macrophage phagocytosis and survive intracellularly. Bone marrow-derived macrophages were infected with opsonized *C. rodentium* WT and Δ*qseC* strains at a MOI=10 and incubated for (A) 30 minutes for phagocytosis analysis or for (B) 3 (C) 6 and (D) 9 hours for intracellular survival**.** CFUs were enumerated by plating serial dilutions**.** Data represent geometric mean ± SD. ns, non-significant differences.

Neutrophils are essential in controlling bacterial infections and host defense against infectious diseases, including *C. rodentium*-mediated disease (114). Our data showed that mice infected with the Δ*qseC* strain presented less severe neutrophil infiltration of the colon (8C). Thus, we hypothesized that the Δ*qseC* strain could be more susceptible to protective neutrophil-mediated responses, and a smaller number of neutrophils would be sufficient to combat the infection caused by Δ*qseC* strain. To

determine whether the QseC sensor enhances *C. rodentium* survival against neutrophils, bone marrow-derived neutrophils were incubated with *C. rodentium* WT or Δ*qseC* strains. After 150 minutes, we evaluated CFUs and the percentage of survival. As a negative control, WT and Δ*qseC* strains were incubated without neutrophils.

We observed that *C. rodentium* is resistant to the bactericidal activity of neutrophils since there were no differences in CFU between the WT strain with or without neutrophils. On the other hand, the Δ*qseC* strain showed a significant CFU reduction compared to the CFU in the control group. In addition, no differences were observed between the WT and Δ*qseC* strains in the control group, indicating that the reduction in the CFU of the Δ*qseC* strain during co-incubation with neutrophils was not due to a defect in Δ*qseC* strain growth or opsonization (Figure 10A). In addition, the survival rate of Δ*qseC* incubated with neutrophils was significantly decreased compared to the WT strain during the same conditions. The WT strain had a survival rate of 83%, while the Δ*qseC* strain demonstrated only 50% (Figure 10B).

Next, we investigated whether an extended incubation time of neutrophils with *C. rodentium* strains could recover the survival of the Δ*qseC* strain. Therefore, we increased the incubation time to 240 minutes. Compared to the 150-minute time-point, the survival after 240 minutes was the same. Similarly, a reduced recovery of CFU was observed in the presence of neutrophils (Figures 10C and D). Together, these results indicate that signaling through the QseC sensor is essential for *C. rodentium* resistance against the bactericidal activity of neutrophils.

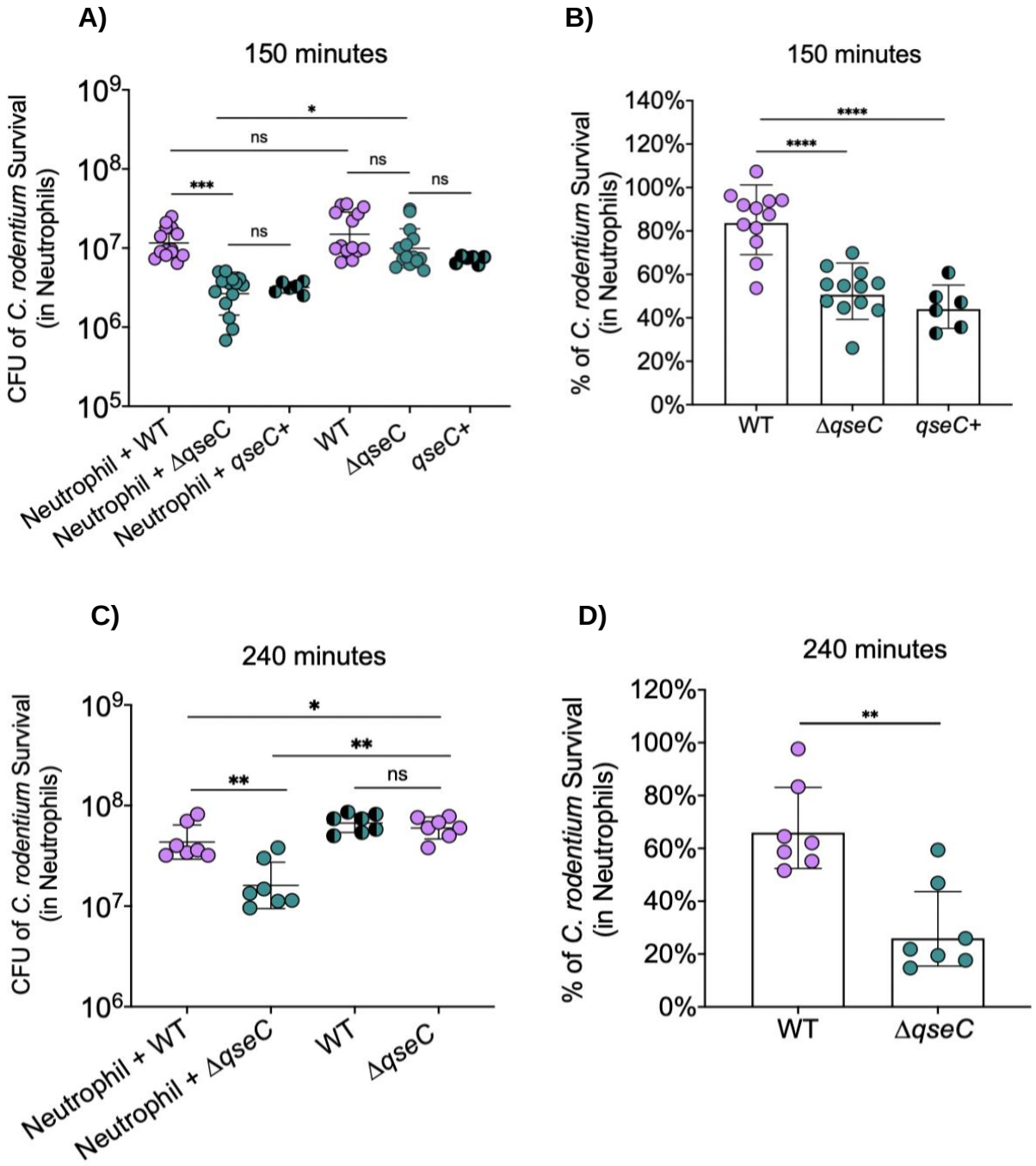

Figure 10. QseC sensor protects *C. rodentium* from antibacterial neutrophil responses. Bone marrow-derived neutrophils were infected with opsonized *C. rodentium* WT, Δ*qseC,* Δ*qseC /qseC*+ strains at a MOI=10 and incubated for 150 or 240 minutes at 37ºC**.** Opsonized *C. rodentium* WT and Δ*qseC* strains were incubated at the same conditions without neutrophils for survival control. CFUs were enumerated by plating serial dilutions**.** The survival percentage was calculated by dividing the CFU from bacteria incubated with neutrophils by the CFU from bacteria without neutrophils and multiplied by 100**.** Data represent geometric mean ± SD. Non-significant differences are indicated by ns; significant differences are indicated by p<0.05 (*), p<0.01 (**), and p<0.0001 (****).

Studies have shown that pathogens use T3SS to target immune cells such as neutrophils to dampen the neutrophil's bactericidal activity and the ability to kill pathogens (139,140). Previously, QseC has been shown to be involved in the regulation of T3SS components of *C. rodentium* during *in vivo* infection (131). Our data suggest that adrenergic signaling mediated by QseC is essential for the survival of *C. rodentium* in neutrophils. To test whether the QseC sensor would play a role in the attenuation of neutrophil responses, we next quantified ROS by flow cytometry and elastase production by ELISA. As shown in Figure 11A, the levels of elastase released by neutrophils infected with *C. rodentium* WT and Δ*qseC* strains were similar. Likewise, we did not observe significant differences in ROS production between neutrophils infected with the WT or Δ*qseC* strains (Figure 11B). These results indicate that QseC is not impairing the activation of neutrophils and that the tested strains are triggering neutrophil responses to the same level.

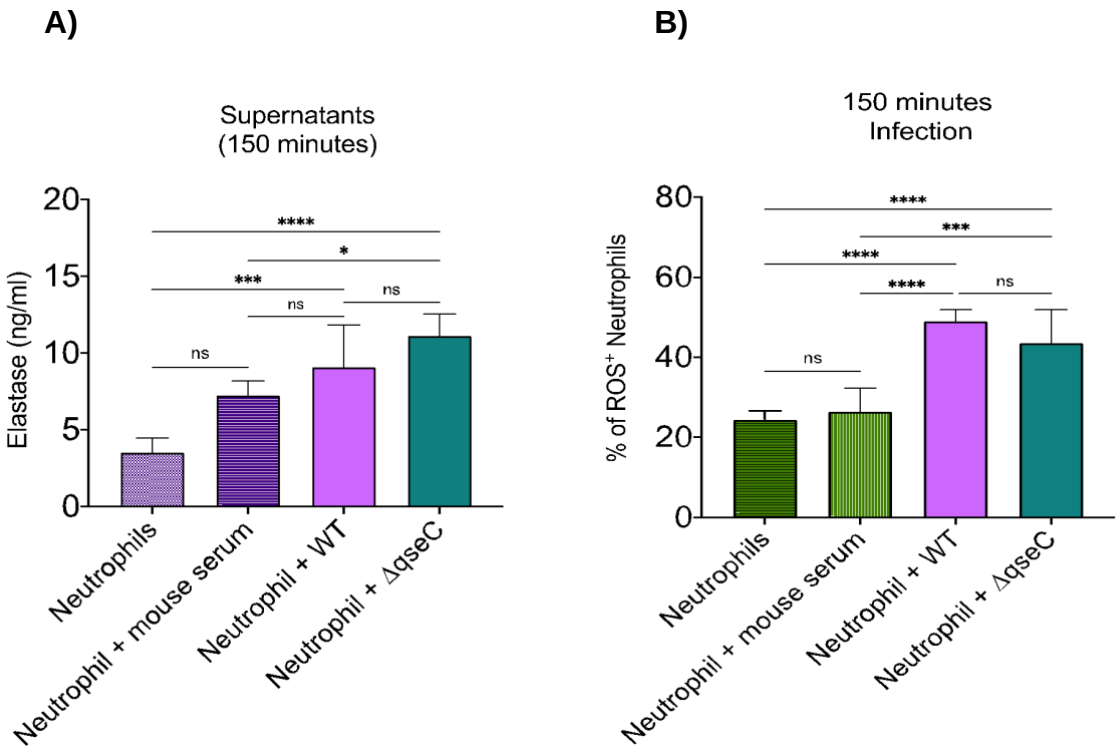

Figure 11. The QseC sensor does not trigger the activation of neutrophils *in vitro*. (**A**) The elastase released by infected neutrophils was detected by ELISA in supernatants. (**B**) ROS production of infected neutrophils was detected by flow cytometry through the conversion of H2DCFDA to fluorescent DCF. Data represent geometric mean ± SD. Data compromised three independent experiments of the groups of neutrophils (n=3), neutrophils + serum (n=3), neutrophils + WT (n=8), and neutrophils + Δ*qseC* (n=8). Non-significant differences are indicated by ns; significant differences are indicated by p<0.05 (*), p<0.01 (**), and p<0.0001 (****).

Since the sensor QseC was not involved in mechanisms that affected the activation of neutrophils but still improved the survival of the WT strain, we next evaluated whether the susceptibility of the Δ*qseC* strain would be due to decreased resistance to neutrophil bactericidal activity. To test the role of the *C. rodentium* QseC sensor for resistance to reactive oxygen species, we isolated neutrophils from B6.129S-*Cybb*$^{tm1Din/J}$ mice, followed by infection with WT and Δ*qseC* strains for 150 minutes. B6.129S-*Cybb*$^{tm1Din/J}$ mice lack the NADPH oxidase type 2 (NOX2)-dependent phagocyte superoxide production. The WT strain exhibited improved survival when incubated with neutrophils lacking an effective ROS response (Figure 12A). The survival rate was 100%,

which was 17% higher than ROS-producing neutrophils (Figure 10A). Nevertheless, Δ*qseC* strain was not rescued by neutrophils lacking ROS production since its survival was decreased in comparison to the WT neutrophils (Figure 12A).

In addition to the evaluation of ROS, we decided to test the role of another important antimicrobial component of neutrophils by isolating neutrophils from calprotectin-knockout (C57BL/6 *S100a9*$^{-/-}$) mice. Calprotectin is an important antimicrobial protein produced by neutrophils that is known to prevent bacteria from acquiring metals, thus inducing bacterial killing (141–143). As shown in Figure 12B, the Δ*qseC* strain survival was not rescued by the absence of calprotectin, demonstrating the same phenotype as for *Cybb*$^{tm1Din/J}$ mice. Together, our results suggest that the QseC sensor of *C. rodentium* is not increasing resistance to ROS or calprotectin responses from neutrophils.

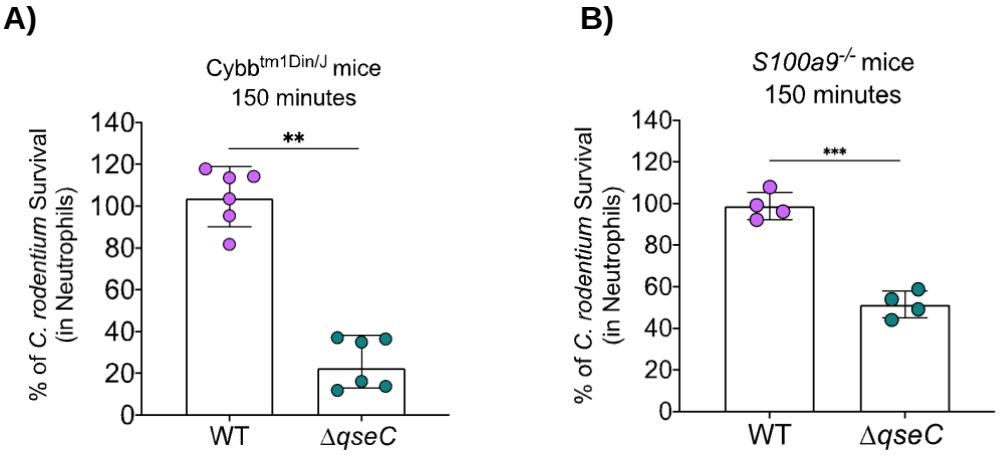

Figure 12. The resistance promoted by the QseC sensor to neutrophils is not dependent on ROS or Calprotectin. Bone marrow-derived neutrophils from C57BL/6129S-Cybb$^{tm1Din/J}$ and C57BL/6 *S100a9*$^{-/-}$ were infected with opsonized *C. rodentium* WT and Δ*qseC* strains at an MOI=10 and incubated for 150 minutes at 37ºC**.** Opsonized *C. rodentium* WT and Δ*qseC* strains were incubated at the same conditions without neutrophils as survival control. CFU were enumerated by plating serial dilutions**.** The percentage of survival was calculated by dividing the CFU from bacteria incubated with neutrophils by CFU from bacteria without neutrophils and multiplied by 100**.** Data compromised two independent experiments. Data represent geometric mean ±SD. The significant differences are indicated by $p<0.01$ (**), and $p<0.001$ (***).

Since our data showed that QseC is not involved in the resistance of *C. rodentium* to ROS or Calprotectin, we next aimed to test other mechanisms of neutrophils that could be involved in the susceptibility observed. First, we analyzed whether QseC signaling would affect *C. rodentium* resistance to NETs, an essential antimicrobial response of neutrophils. Therefore, we incubated neutrophils and WT and Δ*qseC* strains with DNase I. Dnase I was added to hydrolyze the extracellular DNA, which is the backbone of NET formation (144). The WT strain exhibited enhanced survival compared to its group in the presence of DNase I, indicating that NETs might be advantageous for *C. rodentium* survival. The Δ*qseC* showed a similar survival profile either in the presence or absence of DNAse I (Figure 13A). A significant reduction in Δ*qseC* survival was observed compared to the WT in the absence of DNAse I. However, in the presence of this enzyme, the previously observed differences were not seen anymore. Nevertheless, the absence of significant differences was not due to a rescue in Δ*qseC* survival; rather, it was associated with a decrease in WT survival in the presence of DNAse I.

Aminoguanidine (AG) is known as an inhibitor of inducible nitric oxide synthase (iNOS), an enzyme that produces nitric oxide (NO) (145). It is often used in research to investigate the role of nitric oxide in cellular processes and its potential impact on various cell types (145–147). Thus, we decided to investigate the NO effects on Δ*qseC* strain survival. The presence of AG was not enough to recover Δ*qseC* strain phenotype to the WT level since it still presented a significant reduction in comparison to the WT (Figure 13B). Taken together, these data indicate that it is not by the single response of nitric oxide or NET formation that neutrophils are inducing the increased bacterial killing observed for the Δ*qseC* strain.

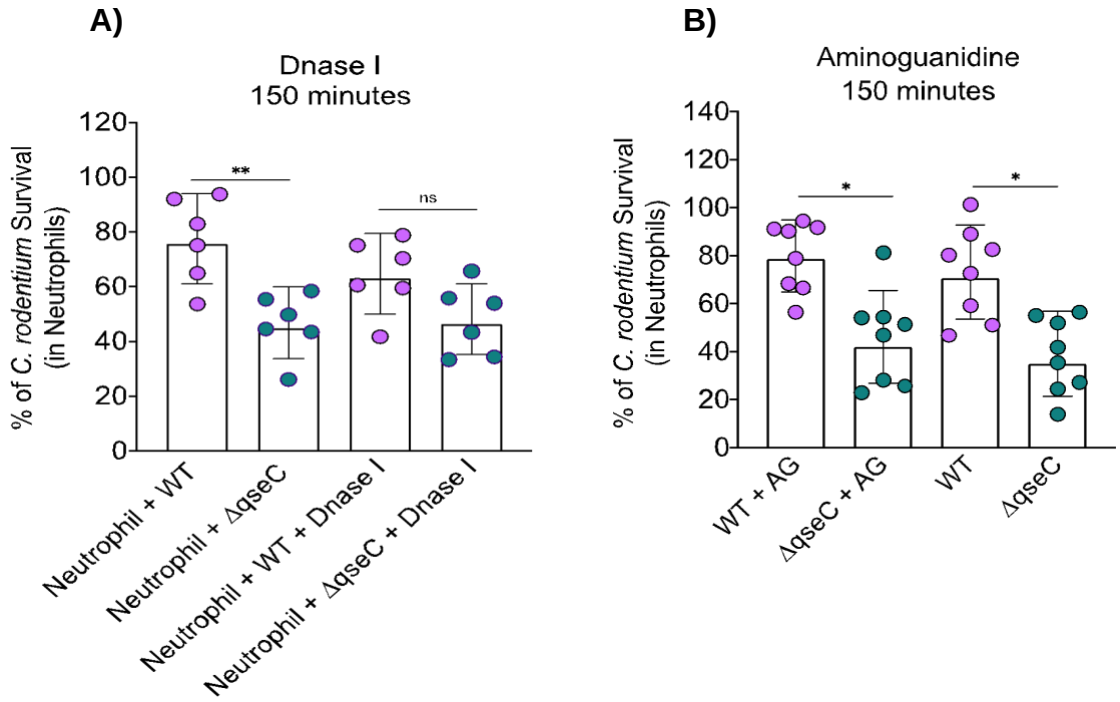

Figure 13. The resistance promoted by the QseC sensor to neutrophils is not dependent on DNAse I or Nitric Oxide. Bone marrow-derived neutrophils were infected with opsonized *C. rodentium* WT and Δ*qseC* strains at a MOI=10 and incubated for 150 minutes at 37 ºC in the presence or absence of DNAse I (200 U/mL) or Aminoguanidine (AG) 1mM/mL**.** Opsonized *C. rodentium* WT and Δ*qseC* strains were incubated at the same conditions with neutrophils without Dnase I or AG for survival control. The percentage of survival was calculated by dividing the CFU from bacteria incubated with neutrophils by the CFU from bacteria without neutrophils and multiplied by 100**.** Data compromised three independent experiments. Data represent geometric mean ± SD. Non-significant differences are indicated by ns; significant differences are indicated by $p<0.05$ (*), $p<0.01$ (**), and $p<0.0001$ (****).

Thus far, we tested various individual responses induced by neutrophils in an attempt to explain the susceptibility shown by Δ*qseC* strain to these cells. Nevertheless, our data suggested that the decrease in Δ*qseC* strain survival was not attributable to a single neutrophil response evaluated by this study. Consequently, we wondered whether the phenotype observed for the Δ*qseC* strain may not result from an antimicrobial response of neutrophils that decreases Δ*qseC* strain survival. Rather, this strain could be more susceptible due to a lack of mechanisms that increase its resistance to the bacterial killing of neutrophils, such as we observed for the WT strain.

Various mechanisms contribute to bacterial resistance against immune cells, and one of these mechanisms involves alterations in membrane organization or composition to enhance resistance against the host's defense mechanisms, such as the complement system and antimicrobial proteins (148). The LPS is an important component found in the outer membrane of Gram-negative bacteria. Studies have shown that LPS plays an important role in pathogen resistance since its modification could affect bacterial recognition and complement activation by immune cells (149–153). Since Δ*qseC* strain is more susceptible to the bacterial activity of neutrophils, we hypothesized that the QseC sensor could be involved in membrane assembly that improves *C. rodentium* resistance to the antibacterial response of neutrophils, especially through LPS modification.

Thus, we performed an LPS evaluation of the *C. rodentium* strains. As shown in Figure 14, there is a significant visual difference in the LPS composition between the WT and Δ*qseC* strains. The WT strain presents three prominent LPS bands comparable to the control LPS of *E. coli*. On the contrary, the Δ*qseC* strain lacks the short chain of the O-antigen. Besides, complementation with a plasmid encoding QseC did not rescue the band, suggesting that the regulatory mechanisms of QseC activation are complex. These data confirm that the QseC sensor regulates the LPS layer of the *C. rodentium* membrane.

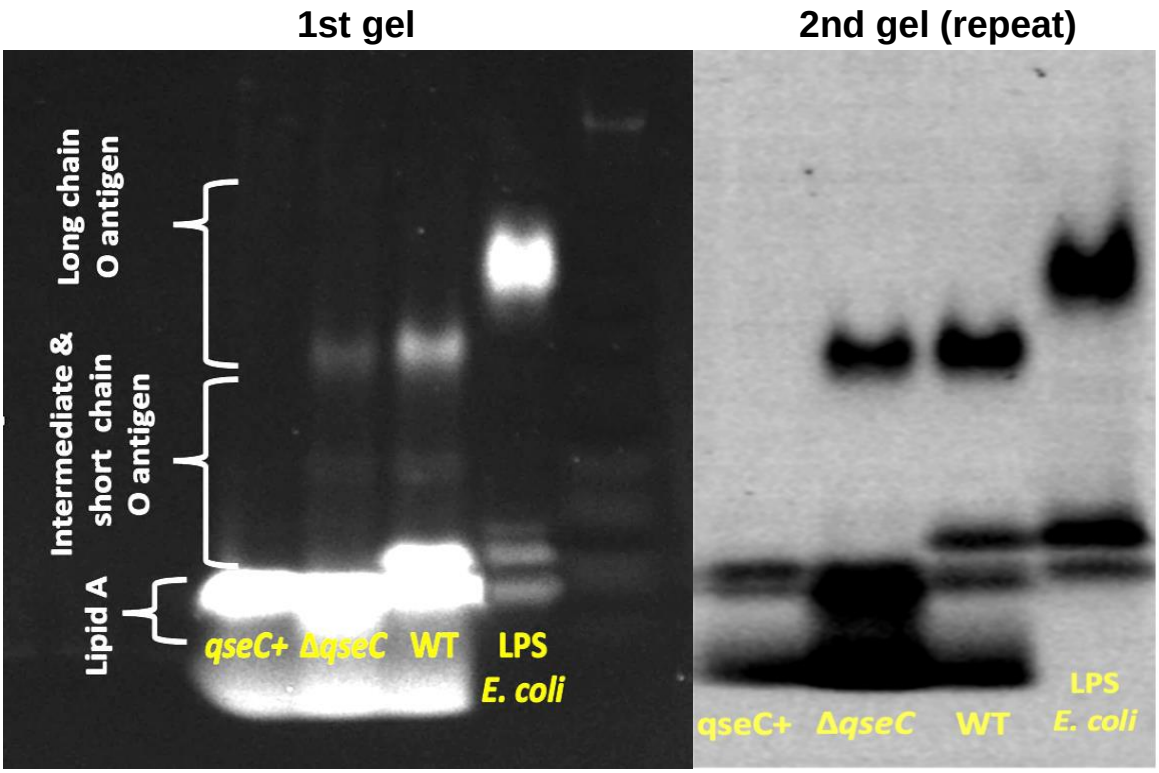

Figure 14. Gel electrophoresis of LPS from *C. rodentium* WT, Δ*qseC,* Δ*qseC/qseC*+ strains. The LPS of *Escherichia coli* was used as a control.

Together, our data indicate that the susceptibility presented by the Δ*qseC* strain does not seem to be dependent on a specific response to neutrophils since the absence of ROS, NETs formation, calprotectin, and nitric oxide alone did not rescue the Δ*qseC* strain survival to the WT levels. Besides, there is a significant difference in the LPS composition between the WT and Δ*qseC* strains.

Opsonization is an important process that increases the phagocytosis of pathogens (154). In addition, it is well known that the complement system also induces damage in the membrane of bacteria by creating pores in their membrane (155). Hence, we decided to perform a similar experiment we did for neutrophils, but instead of using these cells, we exploited the bacterial activity of H_2O_2 to test our hypothesis that the QseC sensor modulates the LPS, thus increasing *C. rodentium* resistance to antimicrobial

responses. As a control, we used non-opsonized bacteria in the presence of H_2O_2. As shown in Figure 15A, no killing in the presence of H_2O_2 was observed for the WT and Δ*qseC* in the non-opsonized group, which suggests that Δ*qseC* strain is not susceptible to the bactericidal activity of H_2O_2. Nevertheless, we noticed a significant reduction in the CFU of Δ*qseC* strain in comparison to the WT in the opsonized group, which was followed by a significant decrease in Δ*qseC* survival rate in comparison to the WT strain (Figure 15B). Together, these data suggest that when in the presence of serum, which contains proteins that harm the membrane of *C. rodentium*, the susceptibility of Δ*qseC* strain to the H_2O_2 bactericidal activity is increased.

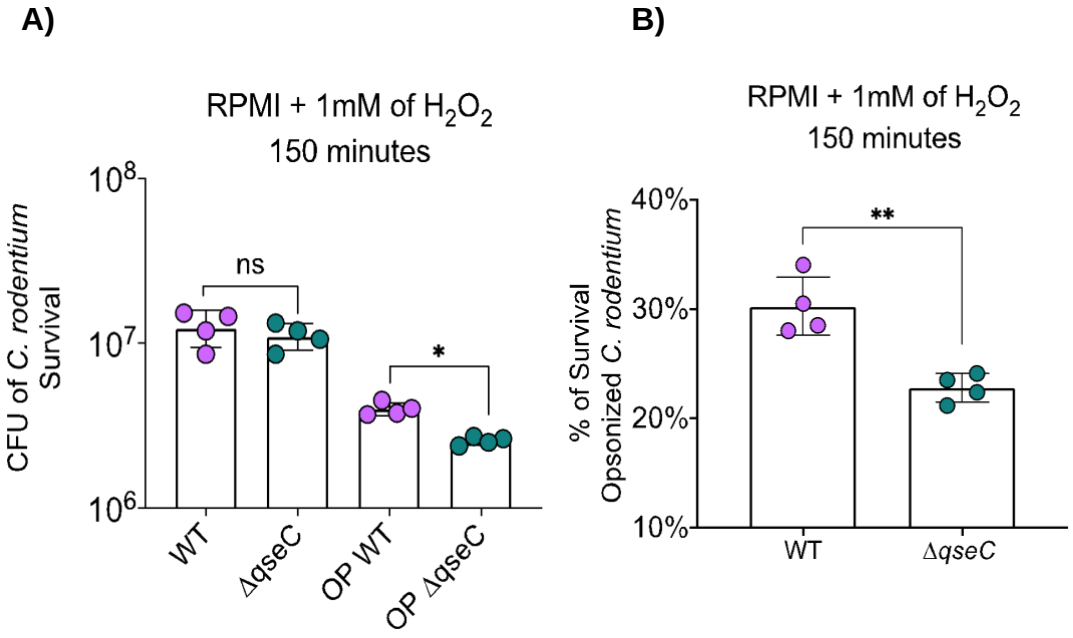

Figure 15. The absence of the QseC sensor increases *C. rodentium's* susceptibility to H_2O_2 in the presence of mice serum. Opsonized (OP) and non-opsonized *C. rodentium* WT and Δ*qseC* strains were incubated in the presence of 1 mM H_2O_2. The percentage of survival was calculated by dividing the CFU from opsonized bacteria by the CFU from non-opsonized bacteria multiplied by 100**.** Data represent geometric mean ± SD. Non-significant differences are indicated by ns; significant differences are indicated by $p<0.05$ (*) and $p<0.01$ (**).

Also, we confirmed that the Δ*qseC* strain is not susceptible to the bacterial activity of H_2O_2. For that, we performed a survival curve of non-opsonized WT and Δ*qseC* strains

in 0.1, 0.6, 1, 5, and 10 mM of H_2O_2. As presented in Figure 16, no difference in *C. rodentium* strain survival was detected between the WT and Δ*qseC* strains in all concentrations of H_2O_2 tested, confirming that Δ*qseC* strain is resistant to the bactericidal activity of H_2O_2 without serum even at higher concentrations.

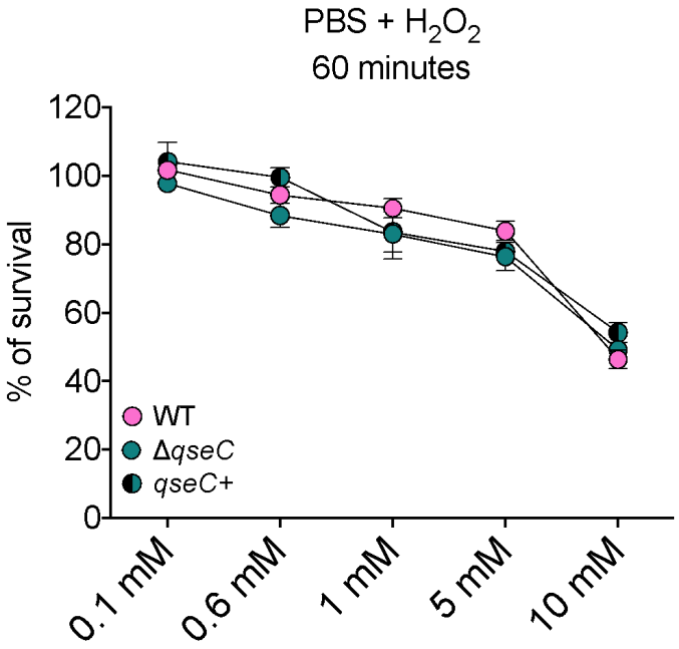

Figure 16. Survival curve of *C. rodentium* in H_2O_2. The *C. rodentium* strains WT, Δ*qseC*, and Δ*qseC/qseC*+ were treated with 0.1 mM, 0.6 mM, 1 mM, 5 mM, and 10 mM of H_2O_2 for one hour in PBS. The percentage mean of survival was calculated by dividing the CFU from bacteria incubated with H_2O_2 in PBS 1x by CFU from bacteria without H_2O_2 and multiplied by 100**.** Data compromised three independent experiments (n=3 each strain). Data represent geometric mean ±SD. Non-significant differences are indicated by ns.

Since the QseC sensor is essential for *C. rodentium* to resist neutrophil bacterial killing, we decided to test whether this phenotype was exclusive to *C. rodentium* or whether other pathogens could also have a similar susceptibility. Thus, we incubated *Salmonella enterica* serovar Typhimurium WT, Δ*qseC,* and Δ*qseC/qseC*+ strains with neutrophils to evaluate their survival profile. As shown in Figure 17, there is significant difference between the STm WT and STm Δ*qseC* strains survival. STm Δ*qseC* had a

significant reduction in comparison to the WT strain. These data suggest that QseC sensor could also be involved in the resistance of other pathogens to neutrophil-killing responses. Thus, we performed LPS analysis for *S.* Typhimurium SL1344 strains to analyze whether *S.* Typhimurium could also have differences in LPS composition. In our gel we did not observe the same short O-antigen band profile as we observed for *C. rodentium*. Besides, the QseC sensor seems to have some modulation in the LPS, since the complemented strain Δ*qseC/*qseC+, where the sensor was being induced, showed more production of the long O-antigen*.* Nevertheless, there were no differences in LPS layers between *S.* Typhimurium WT and Δ*qseC* strains under LB growth conditions.

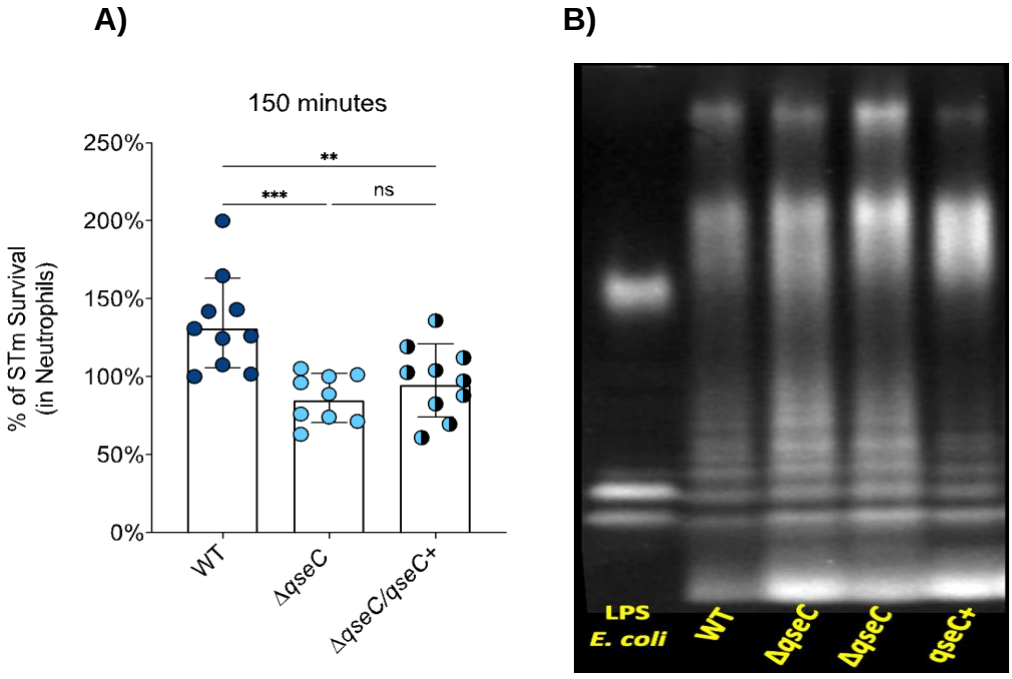

Figure 17. QseC sensor increases *S.* Typhimurium survival from antibacterial neutrophil responses. (A) Bone marrow-derived neutrophils were infected with opsonized *Salmonella enterica* serovar Typhimurium SL1344 WT, Δ*qseC* Δ*qseC*/*qseC*+ strains at a MOI=10 and incubated for 150 at 37 ºC**.** Opsonized *S.* Typhimurium strains were incubated at the same conditions without neutrophils for survival control. CFU were enumerated by plating serial dilutions**.** The survival percentage was calculated by dividing the CFU from bacteria incubated with neutrophils by CFU from bacteria without neutrophils and multiplied by 100**. (B)** Gel electrophoresis of LPS from *S.* Typhimurium WT, Δ*qseC,* Δ*qseC/qseC*+ strain. The LPS of *Escherichia coli* was used as a control. Data represent geometric mean ± SD. Non-significant differences are indicated by ns; significant differences are indicated by p<0.01 (**) and p<0.0001 (***).

The migration of neutrophils to the lamina propria protects mice from dissemination of *C. rodentium in vivo* (156). Our data in mice suggest that without QseC signaling, *C. rodentium* exhibits impaired colonization and dissemination *in vivo*. Since the QseC mutant demonstrated susceptibility to neutrophils, we next tested whether a reduction of neutrophils in the infected tissue would facilitate the systemic spreading of the Δ*qseC* strain. CXCR2 is an important receptor involved in the egress of neutrophils from the bone marrow to peripheral blood and the migration of neutrophils to inflamed tissue (68). Thus, we injected mice with anti-CXCR2 neutralization antibodies on D-1 and D3 post-infection to block the egress from the blood and the recruitment of neutrophils to the infected tissue.

The reduction of neutrophils in the colon during infection after the anti-CXCR2, but none of the other cell types analyzed in this study such as T cells, APCs F4/80+, and CD11c+CD11b+, was confirmed, as shown in Figure 18.

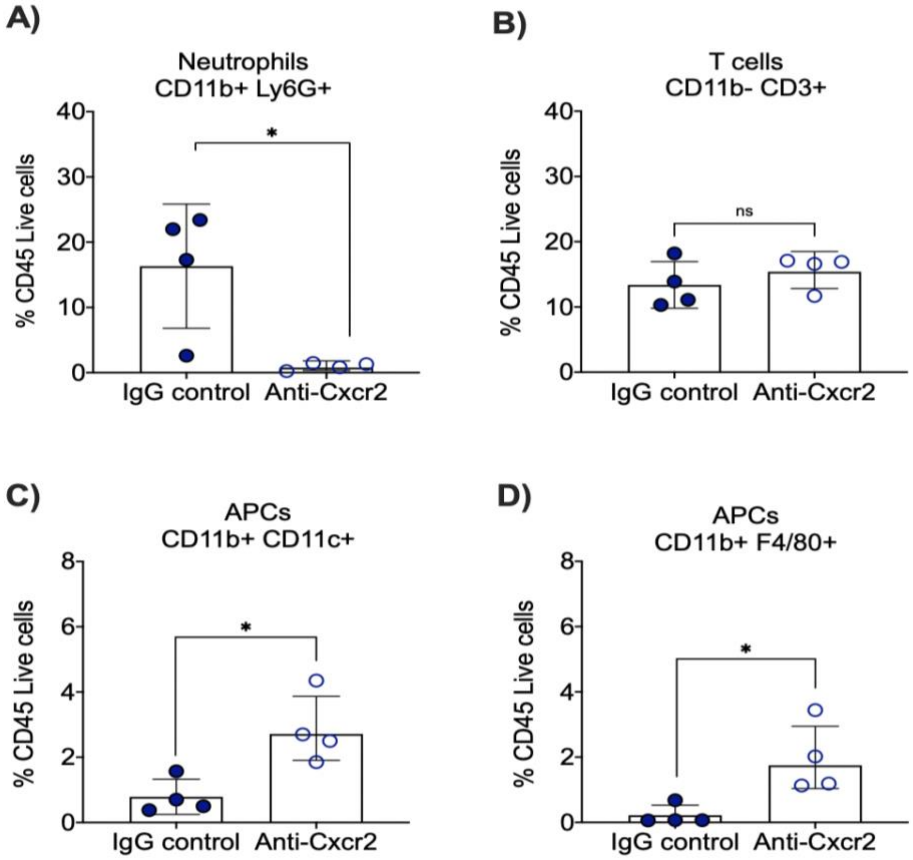

Figure 18. Analysis of immune cells isolated from the colon of C57BL/6 mice that received anti-cxcr2 treatment during infection with *Citrobacter rodentium*. Immune cells isolated from the colon of infected mice after 7 days of *C. rodentium* infection. The frequency of neutrophils (CD45+ CD11b+Ly6G+), Antigen Presenting Cells (APCs) positive for CD45+ CD11b+F4/80+ and CD45+ CD11b+CD11c+, and T cells (CD45+ CD11b-CD3+) from colon live immune cells were determined by flow cytometry. Data compromised two independent experiments. Data represent geometric mean ± SD. Each circle represents an individual mouse. Ns indicate non-significant differences; the significant differences are indicated by $p<0.05$ (*).

Neutralization of CXCR2 facilitated the colonization of the WT strain, yet colonization of the Δ*qseC* strain was not recovered to the level of the WT (Figure 19A). Mice infected with the WT strain and lacking CXCR2-mediated neutrophil responses had an accentuated weight loss compared to IgG-control mice (Figure 19B), suggesting an important function of the QseC sensor for *C. rodentium* virulence during infection. In addition, we observed that anti-CXCR2-treated mice infected with the Δ*qseC* strain displayed a similar level of weight loss compared to IgG control mice infected with the WT

C. rodentium (Figure 19B). This suggests that the Δ*qseC* strain is more virulent in mice lacking CXCR2-mediated neutrophil responses.

The ability of *C. rodentium* to disseminate to systemic sites in the IgG control mice (Figure 19C) and in WT mice (Figure 5) was impaired in the Δ*qseC* strain, as we did not detect CFU in the spleens of these mice. Remarkably, in the absence of the CXCR2-mediated neutrophil responses, the Δ*qseC* strain was detected in the spleen, although not at the same level as the WT strain in the same condition (Figure 19C). Since the dissemination of the Δ*qseC* strain was partly recovered in these mice, we next evaluated the histopathology of anti-CXCR2-treated mice. The lack of CXCR2-mediated responses was insufficient for the Δ*qseC* strain to induce histological colitis scores that we observed for the WT strain in IgG control and anti-CXCR2-treated mice (Figure 19D, 20A, 20B, and 20C). These results indicate that the dissemination observed was not due to increased epithelial injury induced by the Δ*qseC* strain.

Since our results suggested that neutrophils have a role in protecting the host from *C. rodentium* dissemination, we investigated the role of QseC for dissemination in another neutrophil depletion model. To do this, mice were injected with neutralizing anti-Ly6G antibodies at D-1 and D3 post-infection. Similar to the results from mice treated with CXCR2 antiserum, systemic dissemination of the Δ*qseC* strain was facilitated in mice following anti-Ly6G treatment, although gut colonization remained lower (Figure 21A and B).

Dampened Immune responses by blockage of neutrophil migration through CXCR2 or the neutrophil neutralizing antibody anti-Ly6G did not increase the colonization and gut inflammation of the Δ*qseC* strain, although its systemic spreading is facilitated.

Overall, these results suggest that the QseC sensor may be involved in mechanisms that aid *C. rodentium* to overcome mucosal protection by neutrophils and to spread systemically and confirm that QseC sensor mediated-signaling is essential for *C. rodentium* to effectively colonize the gut and to induce pro-inflammatory responses in mice.

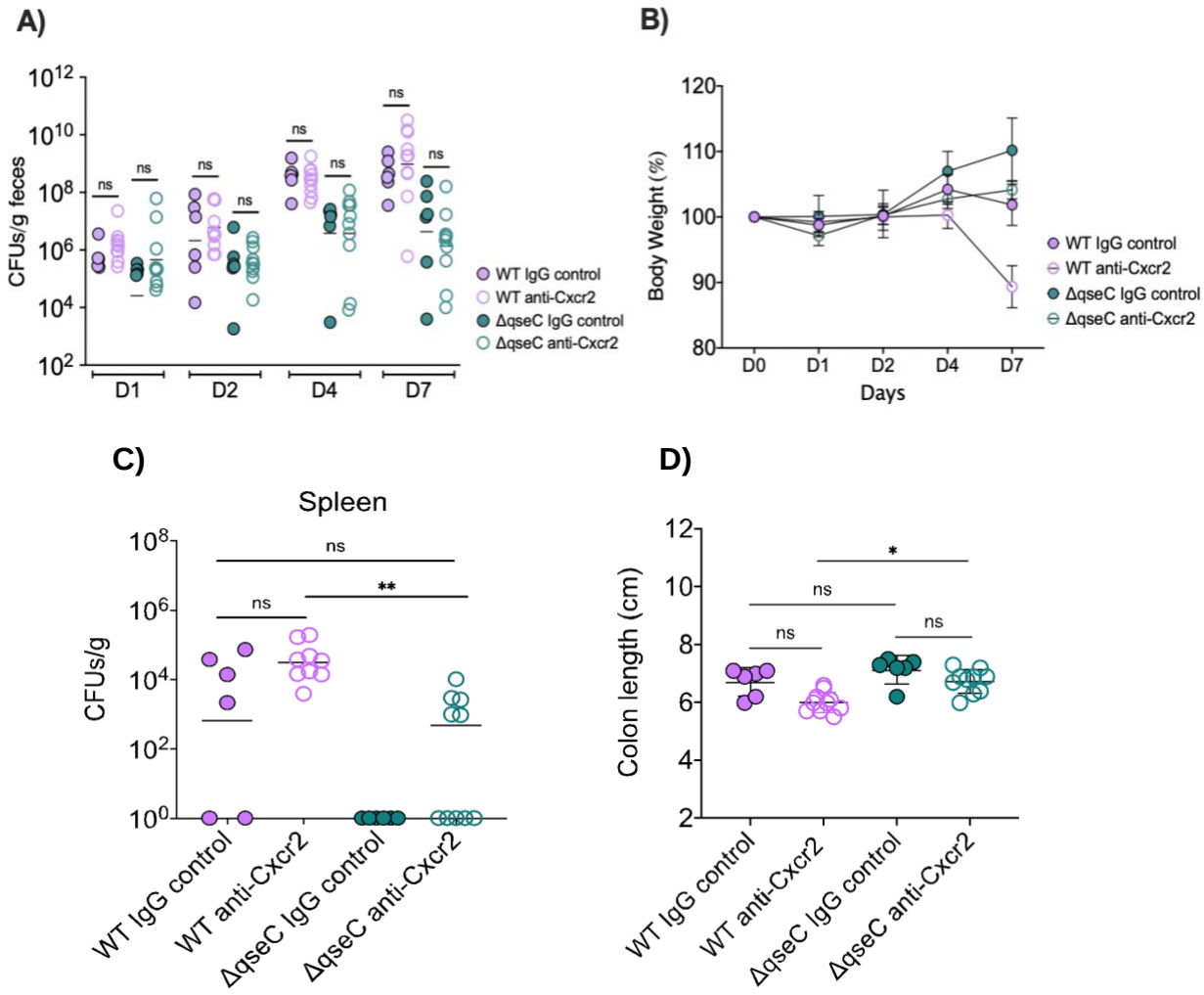

Figure 19. The lack of CXCR2-mediated responses facilitates the spreading of the *ΔqseC* strain. Mice received anti-CXCR2 neutralization antibodies intraperitoneally on D-1 and D4 post-infection**.** IgG isotypes were used for the control group**. (A)** Differences between WT and ΔqseC strains CFUs in fecal samples on days 1-, 2-, 4- and 7-day (D1, D2, D4, and D7) post-infection in WT mice. **(B)** Body weight between WT infected mice with *C. rodentium* WT or ΔqseC strains at D1, D2, D4, and D7 post-infection. **(C)** *C. rodentium* CFUs in the spleen of infected mice seven days post-infection (D7). **(D)** Colon length was measured at D7 post-infection. Data represent geometric mean ±SD. Each circle represents an individual mouse. Non-significant differences is indicated by ns; significant differences are indicated by $p<0.05$ (*), $p<0.01$ (**), and $p<0.0001$ (****).

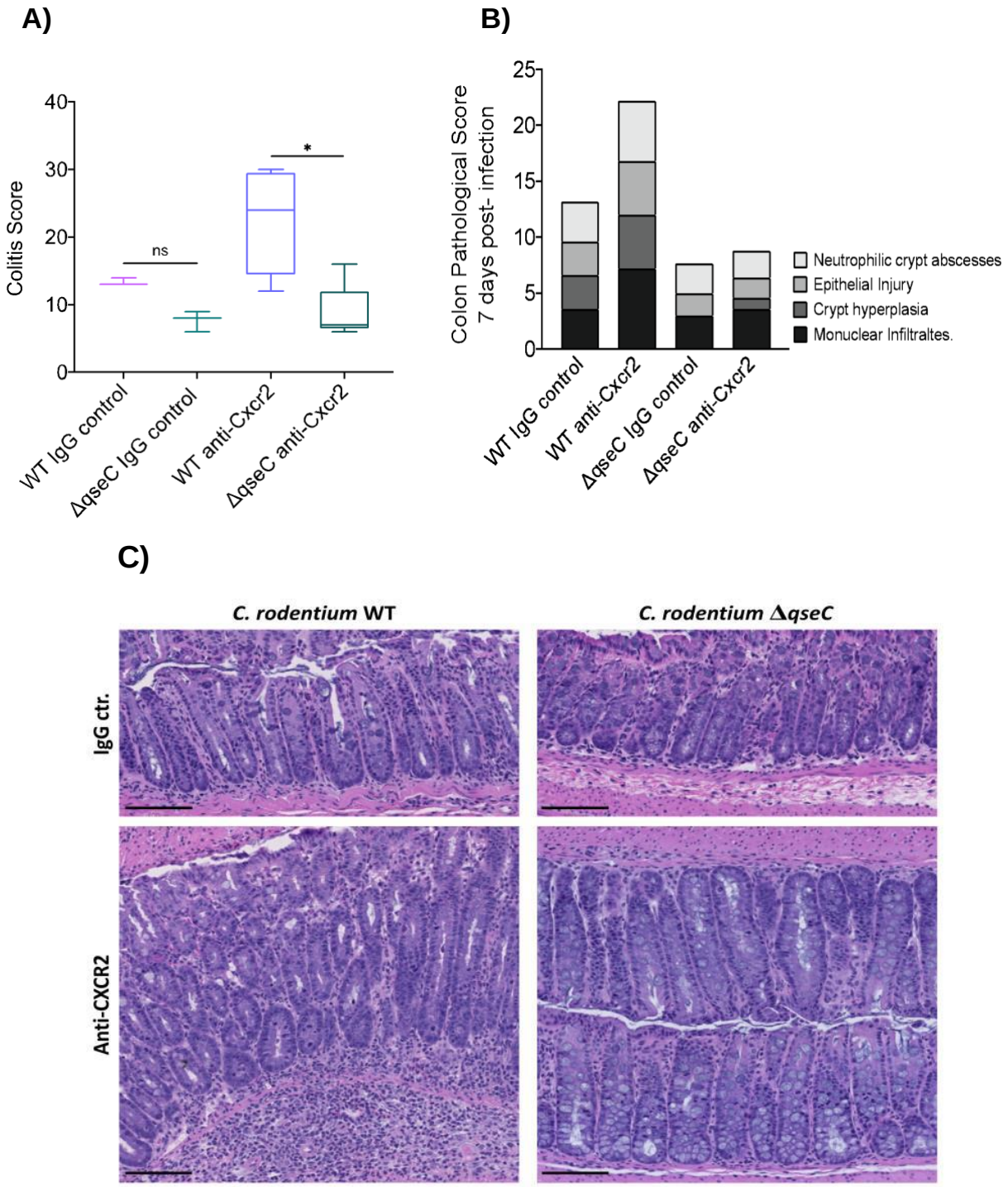

Figure 20. Histopathology from mice treated with IgG control or anti-CXCR2 C57BL/6 and infected with *Citrobacter rodentium* WT and Δ*qseC* strains. (A) Colitis score at day 7 post-infection of IgG control mice infected with *C. rodentium* WT (n=3) and Δ*qseC* (n=3) and anti-Cxcr2 infected with *C. rodentium* WT (n=5) and Δ*qseC* (n=5)**. (B)** Colon pathological scores 7 days post-*C. rodentium* infection: **(C)** Representative H&E stained colon sections from mice infected with WT or Δ*qseC C. rodentium*. Bars represent the geometric mean ±SD. Significant differences are indicated by p<0.05 (*).

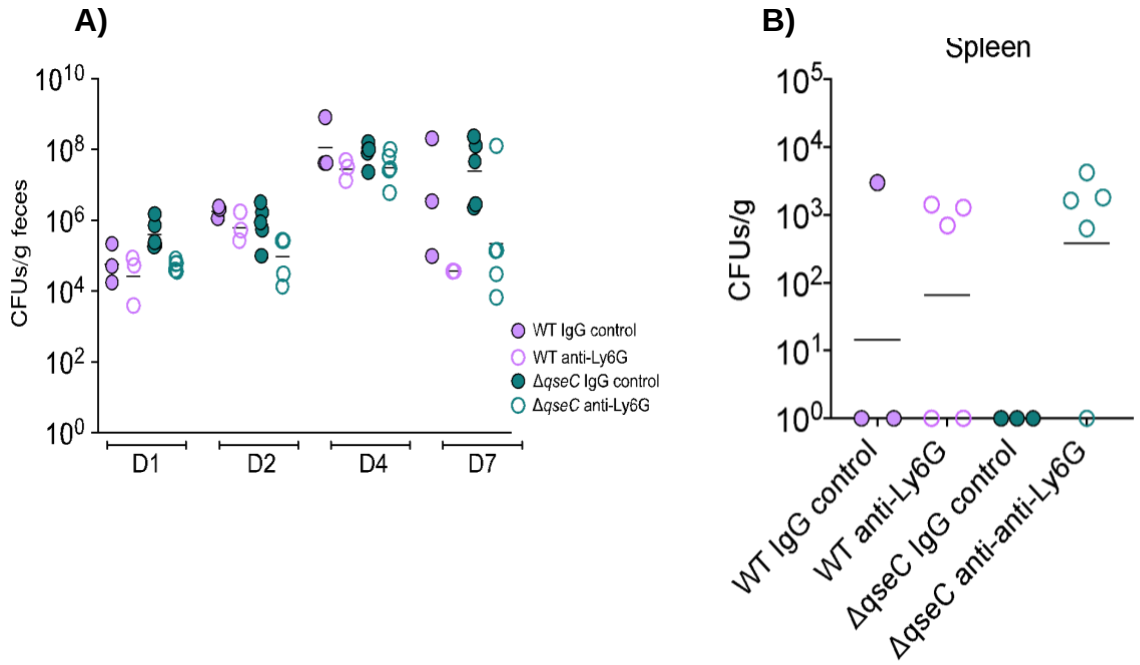

Figure 21. Dissemination of *C. rodentium* to systemic sites depends on evading neutrophils via QseC sensor-mediated responses. Mice received anti-Ly6G neutralizing antibodies intraperitoneally D-1 and D4 post-infection**.** IgG isotypes were used for the control group**. (A)** Differences between WT and Δ*qseC* strains CFUs in fecal samples on days 1-, 2-, 4- and 7-day (D1, D2, D4, and D7) post-infection in WT mice. **(B)** *C. rodentium* CFUs in the spleen of infected mice seven days post-infection (D7). Data represent geometric mean ± SD. Each circle represents an individual mouse. Non-significant differences are indicated by ns; the significant differences are indicated by $p<0.05$ (*), $p<0.01$ (**), and $p<0.0001$ (****).

2.4. Discussion

In the gut, invasive bacteria encounter various challenges, including overcoming the microbiota and evading the host's immune response. The success of infection relies on the pathogen's ability to sense environmental changes and coordinate gene expression to activate virulence mechanisms at the appropriate time (46). Epinephrine and norepinephrine play an essential role in host homeostasis and physiology. Enteropathogens such as *C. rodentium* and EHEC evolved mechanisms to sense these neurotransmitters through the QseC sensor and to activate their virulence genes accordingly (126,131). Activation of virulence genes benefits pathogens by improving

host colonization, evading immune responses, and outcompeting commensal microbes, among others.

Our data demonstrated that signaling via the QseC sensor is critical for *C. rodentium* to colonize mice successfully, as the level of Δ*qseC* colonization decreased significantly compared to the WT strain. Furthermore, the QseC sensor is directly correlated to the disease outcome. Mice infected with the WT strain exhibited splenomegaly, which correlated with increased CFU numbers in this organ. In contrast, the Δ*qseC* strain did not disseminate to the spleen of infected mice. The observed attenuation of the Δ*qseC* strain is in agreement with previous studies showing that C3J mice, which otherwise succumb to *C. rodentium* infection, exhibited improved dose-dependent survival following infection with the Δ*qseC* strain (131).

Furthermore, our work revealed that inflammation induced by *C. rodentium* depends on genes regulated by the QseC sensor. Mice infected with the Δ*qseC* strain presented increased colon lengths, which correlated with a reduction in colitis scores compared to the WT strain. Also, our data established that *C. rodentium* could not induce TCCH without a QseC sensor. The observed differences in colonization and inflammation are probably related to a decreased activation of T3SS of *C. rodentium* in the absence of the QseC sensor. Previous studies reported that mice infected with the Δ*qseC* strain had decreased expression of T3SS-related genes, such as *Tir* and *EspA,* in the feces, and signals mediated by norepinephrine and epinephrine increased the expression of T3SS-genes in *C. rodentium* (131). Accordingly, the lack of a functional T3SS impairs the virulence of *C. rodentium* (91,98,99,157).

In addition, colonic infiltration of neutrophils and CD11c+CD11b+ cells triggered by Δ*qseC* was decreased, although the numbers of APCs F4/80+ were similar compared

to mice infected with the WT strain. We found that *C. rodentium* is not dependent on QseC sensor signaling to resist macrophage responses *in vitro*. However, our data suggest that sensing signals through QseC increases the resistance of *C. rodentium* to neutrophil-mediated responses *in vitro*. Thus, the decreased number of neutrophils observed during *in vivo* infection could be due to a more efficient eradication of *C. rodentium* Δ*qseC* by neutrophils, thereby triggering fewer immune cells to infiltrate the colon. Notably, previous studies have shown that human neutrophils synthesize, store, and release epinephrine, which can be sensed by *Aggregatibacter actinomycetemcomitans* via the QseC sensor, thereby promoting bacterial growth (158), this could also be another source of signalization for *C. rodentium* to resist to this cells. Nevertheless, the increased killing observed for the Δ*qseC* strain was not due to ROS, nitric oxide, Calprotectin, or NET formation by neutrophils.

Our data suggest that QseC sensor plays an important role in LPS synthesis in *C. rodentium*, since the Δ*qseC* strain showed differences in the short-chain portion of the O-antigen. Studies have shown that quorum sensing signaling is involved in pathogen's regulation of LPS synthesis (159). For example, *Pasteurella multocida* uses the QseC sensor to regulate genes involved in LPS and capsule biosynthesis. Besides, a study showed that the deletion of the QseC sensor in *Francisella tularensis* decreased its resistance to the bactericidal activity of normal serum (160). In our study, *C. rodentium* Δ*qseC* did not show impaired resistance to the mouse serum in the concentration used. Besides, no differences in survival were observed in the presence of H_2O_2. Nevertheless, the presence of H_2O_2 in the opsonized Δ*qseC* strain led to a significant reduction in Δ*qseC* survival. Thus, the differences in the WT and Δ*qseC* strain survival observed in our *in vitro* experiments with neutrophils may be due to modifications of *C. rodentium* LPS

dependent on the QseC sensor that, together with the action of the complement system, increase *C. rodentium* Δ*qseC* strain susceptibility to neutrophils bactericidal responses.

Moreover, we investigated the ability of CXCR2-dependent neutrophil migration to understand the role of the QseC sensor for *C. rodentium* in mice with an impairment in this immune cell-mediated response. Previous studies have shown that CXCR2-mediated responses protect mice against *C. rodentium* infection (114). Our data support these results since anti-CXCR2-treated mice infected with the WT strain presented with higher colitis scores, increased systemic dissemination, and more weight loss. Moreover, our data revealed that even in the absence of a CXCR2-mediated response, the Δ*qseC* strain was unable to recover its colonization to the level of the WT strain, which could be due to the colonization resistance promoted by the microbiota.

Although we did not observe differences in the colonization and inflammation for the Δ*qseC* strain in mice treated with anti-CXCR2, *C. rodentium* Δ*qseC* was able to disseminate to the spleen. Because our data showed that the absence of the QseC sensor decreases *C. rodentium* survival in neutrophils, and previous studies have reported that lamina propria neutrophils are essential to protect the host from *C. rodentium* dissemination (156), the absence of neutrophils could facilitate the systemic spreading of the Δ*qseC* strain. Moreover, our pilot experiments with anti-Ly6G support these data since spreading to systemic sites was comparable to that observed in anti-CXCR2-treated mice.

Future experiments will help to understand how QseC improves the survival of *C. rodentium* and the assembly of the O-antigen, thus providing resistance to neutrophil responses, and whether the detection of neutrophil-derived epinephrine has a role in this process. Altogether, these data demonstrate that QseC has an important role in A/E

pathogens virulence and blocking this sensor could be a powerful strategy to combat A/E pathogens infections, especially when antibiotic treatment is not recommended, such as for EHEC infections.

Chapter III: The role of interleukin-22 for mucosal immune defenses against *Citrobacter rodentium*

3.1. Introduction

Infections caused by bacterial enteropathogens have been associated with severe disease and mortality worldwide (161). Among diarrheal pathogens, attaching and effacing (A/E) enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) stand out as major public health concerns (79,87). Because mice exhibit relatively high resistance to EHEC and EPEC infection, the mouse pathogen *Citrobacter rodentium* has become a widely used model for investigating the mechanisms through which A/E pathogens cause disease and for gaining insights into mucosal responses during infection (94,97).

The host depends on signals like cytokines and chemokines produced by intestinal epithelial cells and immune cells to coordinate appropriate responses during infection of enteric pathogens, such as *C. rodentium* (9). Interleukin (IL)-22 is a cytokine that plays a central role in orchestrating various aspects of gut mucosal immunity. It is produced by lymphocyte subsets in response to proinflammatory signals, including IL-23, which is produced by myeloid cells (31). Lymphocytes that secrete IL-22 include Th1, Th22, CD8+ T cells, γδ T cells, natural killer cells, lymphoid tissue inducer cells, and innate lymphoid type 3 cells (ILC3) (38,162).

During infection, IL-22 protects the host by stimulating epithelial cells to produce antimicrobial proteins, including the C-type lectins Reg3β and Reg3γ, which exhibit antimicrobial activity against a subset of bacterial pathogens (39,42,162–165). IL-22 also induces expression of the metal-sequestering antimicrobial proteins calprotectin,

lipocalin-2, and hemopexin (39–41,118,166,167). Additionally, IL-22 has been shown to induce the expression of complement component C3 (166) and to regulate intestinal epithelial barrier function, including gut permeability and regeneration (162).

The protective role demonstrated by IL-22 during infection is context- and pathogen-dependent. For example, the absence of IL-22 does not increase host susceptibility during infection with either *Neisseria gonorrhoeae*, *Listeria monocytogenes*, or *Salmonella enterica serovar* Typhimurium (168–171). In contrast, IL-22 is essential for protecting the host against *Klebsiella pneumoniae* and *C. rodentium* (39,172). Wild-type C57BL/6 mice typically resolve *C. rodentium* infection successfully within weeks post-infection. Conversely, C57BL/6 mice lacking IL-22 (*Il22*$^{-/-}$) succumb to the infection, exhibiting mortality patterns analogous to more susceptible mouse strains, including C3H/6J mice (98,173).

Several IL-22-dependent antimicrobial defenses have been associated with protection during *C. rodentium* infection, including the regulation of antimicrobial proteins (39,118) and gut epithelial function (174,175). In this study, we aimed to investigate the specific contribution of some IL-22-dependent antimicrobial responses to host protection during *C. rodentium* infection.

3.2. Material and Methods

Bacterial strains: The wild-type *Citrobacter rodentium* strain DBS100 (obtained from Prof. Vanessa Sperandio, University of Wisconsin, Madison) was used in this study (176). Bacteria were cultured overnight aerobically in Luria-Bertani (LB) broth containing nalidixic acid (Nal, 50 mg/mL) at 37ºC under rotation. The following day, overnight

bacterial cultures were washed, centrifuged, diluted, and resuspended in 1X Phosphate Buffered Saline (PBS) to a concentration of $5x10^9$ CFU per mouse.

Animal Experiments: For all infection experiments, we used C57BL/6 five-week-old male and female mice. *Il22*$^{-/-}$ (31), *Lcn2*$^{-/-}$ (167), *S100a9*$^{-/-}$ (177), *C3*$^{-/-}$ (JAX stock #029661) (178), *Reg3b*$^{-/-}$ (179) and *Reg3g*$^{-/-}$ (42) mice (all in a C57BL/6 background) were orally gavaged with $5x10^9$ *C. rodentium* in 200 µl of 1X PBS. For *Il22*$^{-/-}$ mice, we used cohoused WT littermate mice as a comparison. For all other colonies, WT mice were purchased from The Jackson Laboratory and cohoused a week before infection and for the duration of the experiment. All mice were free of detectable *Escherichia coli* in their fecal samples, based on MacConkey agar plating. Fecal samples were collected on days 1, 4, 7, 10, and 12 post-infection, homogenized, serially diluted, and plated on LB agar containing Nal for enumeration of colony-forming units (CFU). Mouse survival was followed for 25 days post-infection. A loss of 20% from initial body weight was used as a humane endpoint. Some mice were euthanized at day 12 post-infection for histopathology and gene expression analysis. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, San Diego.

RNA extraction: Colons collected from mice infected with *C. rodentium* or uninfected controls were snap-frozen in liquid nitrogen and stored at -80^{o}C until further processing. Next, the samples were homogenized with mortar and pestle, collected in a tube with Tri-Reagent® (Millipore-Sigma), and vortexed for 5 minutes. The samples were transferred in an Eppendorf tube containing 0.1 mL of 1-Bromo-3-chloropropane (MRC), vortexed, stored at room temperature for 10 minutes, followed by centrifugation at 12,000 x *g* for 15

minutes at 4ºC. Subsequently, the aqueous phase was collected in a 1.5 mL tube with 0.5 mL of isopropanol (Fisher Chemical) and stored for 30 minutes at -20 ºC followed by 12,000 x *g* for 15 minutes at 4ºC. The RNA was washed with 75% ethanol (Fisher Chemical) and centrifuged at 12,000 x *g* for 8 minutes at 4ºC. Finally, the ethanol was air dried, and the RNA pellets were dissolved in DNAse and RNAse-free water (Invitrogen) and quantified.

Analysis of gene expression by RT-qPCR: Reverse transcription of 1 µg of total RNA was performed using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific). Quantitative real-time PCR (qRT-PCR) for gene expression analysis was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). Target gene expression was normalized to β-actin (*Actb*). Fold changes in gene expression were relative to uninfected controls and calculated using the ΔΔCt method. Primers used in the study are listed in Supplemental Table 1.

Table 1 - Primers used in this study

Target	Forward primer (5' – 3')	Reverse primer (5' – 3')	Reference
Cldn2	GGCTGTTAGGCACATCCAT	TGGCACCAACATAGGAACTC	(174)
Ocln	GCTGTGATGTGTGTGAGCTG	GACGGTCTACCTGGAGGAAC	(174)
Cldn1	TCCTTGTTCGGCTATGTGTC	GGCATGCACCTAAGAATCAG	(174)
Fut2	TGCACTGGCCAGGATGAA	GCGCTAGAGCGTTGTGCAT	(175)
Muc2	GCTGACGAGTGGTTGGTGAATG	GATGAGGTGGCAGACAGGAGAC	(176)
Itgb1	AGTGCTCCCACTTCAATCTCACCA	TCTCCTTGCAATGGGTCACAGGAT	(174)
Reg3b	ATGGCTCCTACTGCTATGCC	GTGTCCTCCAGGCCTCTTT	(39)
Reg3g	ATGGCTCCTATTGCTATGCC	GATGTCCTGAGGGCCTCTT	(39)
Il1b	ATGAGAGCATCCAGCTTCAA	TGAAGGAAAAGAAGGTGCTC	(180)
Tnfa	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	(181)
Ifng	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG	(181)
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	(181)
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	(181)
Nos2	TTGGGTCTTGTTCACTCCACGG	CCTCTTTCAGGTCACTTTGGTAGG	(182)
Il22	GGCCAGCCTTGCAGATAACA	GCTGATGTGACAGGAGCTGA	(39)
S100A8	CCATCGCAAGGAACTCCTCGAAG	ACAAGGAAATCACCATGCCCTCTAC	Raffatellu Lab
S100A9	ATTCAGACAAATGGTGGAAGCACAG	CATCAGCATCATACACTCCTCAAAGC	Raffatellu Lab

FITC-Dextran gut permeability assay: Five-week-old male and female *Il22*$^{+/+}$ and *Il22*$^{-/-}$ littermate mice were infected with *C. rodentium* as described. At day 12 post-infection,

mice were gavaged with 100 µl of 80 mg/mL Fluorescein isothiocyanate–dextran (FITC-dextran) with an average molecular weight of 4,000 (Sigma-Aldrich) in 1x PBS. After 4 hours, mice were euthanized, and the blood was collected by cardiac puncture and transferred to Eppendorf tubes containing 0.5M EDTA. Spleen and fecal samples were collected for CFU enumeration. Next, the tubes with blood were centrifuged, and plasma was collected and diluted in a ratio of 1:4 in 1x PBS. PBS was used as a blank to normalize the data. Fluorescence was determined at 530 nm with an excitation of 485 nm on a BioTek Synergy HTX plate reader.

Histopathology. At day 12 post-infection, whole colons from infected WT and *Il22*$^{-/-}$ mice were collected into Swiss rolls, formalin-fixed, paraffin-embedded, and sectioned for histopathology. The following parameters were scored as follows. Mononuclear infiltration scores are 0 for absent (normal sparse lymphocytic infiltrate), 1 for mild (diffuse increase in lamina propria, usually with plasma cells), 2 for moderate (lamina propria increased with basal localization aggregates displacing crypts, distinguish normal MALT), and 3 for severe (lamina propria with submucosal infiltration). Crypt hyperplasia scores are 0 for absence and 3 for the highest score (severe). Epithelial injury scores are 1 for mild (crypt dropout/crypt distortion or surface epithelial damage, no frank erosion or ulceration), 2 for moderate (focal ulceration), and 3 for severe (multifocal or extensive ulceration). Neutrophilic crypt abscesses scores are 0 for absent, 1 for mild (lamina propria only), 2 for moderate (lamina propria plus cryptitis and crypt abscesses), and 3 for severe (sheet-like or submucosal infiltrate).

Statical Analysis. Statistical analysis was performed with GraphPad Prism v9. The Shapiro-Wilk test was used for normality distribution. For normally distributed groups, p-p-values were calculated by unpaired Student's *t*-test. The Mann-Whitney U test was performed for unpaired data that were not normally distributed. One-way and two-way analysis of variance (ANOVA) with multiple-comparison testing were used when analyzing more than two groups. The log-rank test was used to compare survival between groups.

3.3. Results

Previous studies in mice have demonstrated the importance of IL-22 to effectively clear *C. rodentium* infection. *Il22*$^{-/-}$ and *Il22ra1*$^{-/-}$ mice infected with *C. rodentium* exhibit heightened intestinal epithelial damage, increased systemic bacterial burden, and greater mortality (39,118,175). Based on these studies, we decided to investigate whether IL-22 knockout mice in our colony could also display increased susceptibility to *C. rodentium* infection. Thus, we infected C57BL/6 WT and *Il22*$^{-/-}$ with *C. rodentium* and collected fecal samples to analyze colonization levels at days 1, 4, 7, 10, and 12 post-infection and monitored the survival of mice for 25 days post-infection. As shown in Figure 22A, all WT mice infected with *C. rodentium* remained alive 25 days after infection. Moreover, mice recovered from the initial weight loss, and the level of colonization declined (Figure 22B and C). In contrast, *Il22*$^{-/-}$ mice infected with *C. rodentium* infection displayed heightened susceptibility, with almost 100% lethality by day 25 (Figure 22A). Moreover, these *Il22*$^{-/-}$ mice showed significantly higher colonization levels and weight loss compared to WT controls (Figure 22B and C). These findings align with prior studies, providing evidence that *C. rodentium* indeed induces a lethal infection in *Il22*$^{-/-}$ mice in our animal facility.

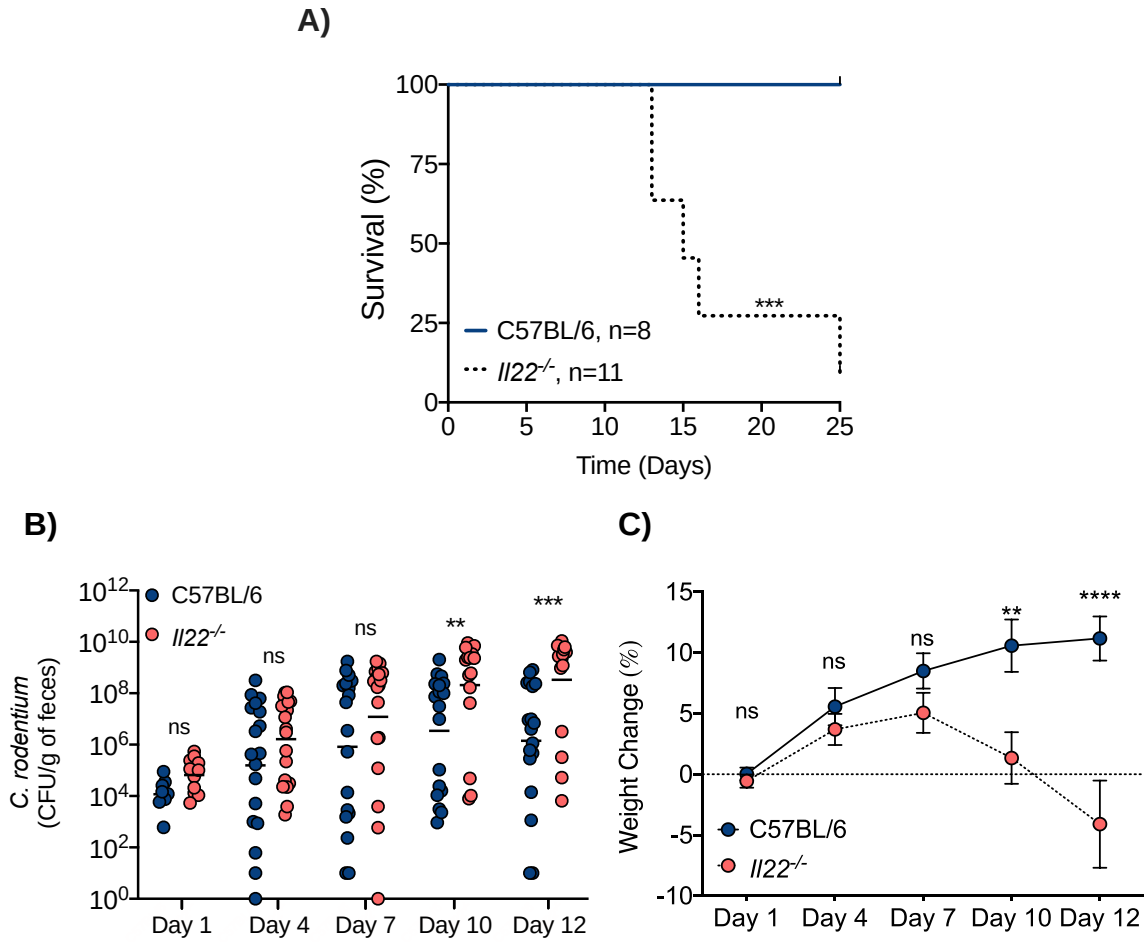

Figure 22. IL-22 confers protection during *C. rodentium* infection. (A) Survival of WT and *Il22*$^{-/-}$ mice infected with *C. rodentium*. **(B)** Fecal CFU of infected mice on days 1, 4, 7, 10, and 12 post-infection. **(C)** Weight course of mice infected with *C. rodentium*. Each circle represents an individual mouse. Bars represent the geometric mean. Significant differences are indicated by $p<0.01$ (**) and $p<0.01$ (***). ns = not significant.

Because IL-22-deficient mice started to succumb to infection beginning on day 12 post-infection (Figure 22A), we selected this time point to analyze the expression of antimicrobial genes that have been shown to be under IL-22 modulation. First, we confirmed that IL-22-deficient mice do not express IL-22 (Figure 23). The expression levels of the antimicrobial proteins *S100a8* and *S100a9* that encode the antimicrobial protein calprotectin were comparable between WT mice and *Il22*$^{-/-}$ mice. The gene that encodes lipocalin-2, *Lcn2,* showed a trend towards decreased expression in *Il22*$^{-/-}$ mice

compared to the WT mice, although the difference was not statistically significant. On the contrary, the expression of C3 (encoding complement component C3), *Reg3y*, and *Reg3β* (encoding the C-type antimicrobial lectins Reg3γ and Reg*β,* respectively) was significantly reduced (Figure 23). Thus, at the time when mice started to succumb to *C. rodentium* infection (day 12), there were differences in the expression levels of certain antimicrobial proteins that may contribute to host protection during *C. rodentium* infection.

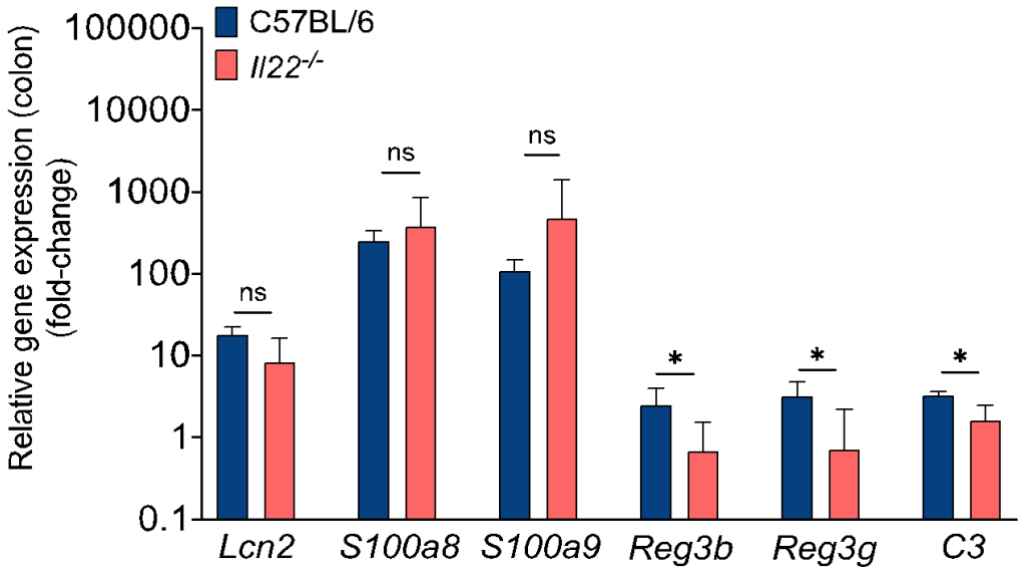

Figure 23. Expression of antimicrobial genes in WT and *Il-22*$^{-/-}$ mice. A real-time polymerase chain reaction (RT-qPCR) was used to determine the expression of *Lcn2*, *S100a8*, *S100a9*, *Reg3g*, *Reg3b,* and *C3* in the colon of WT and *Il22*$^{-/-}$ mice 12 days after infection. WT infected n=4, *Il22*$^{-/-}$ infected n=4, *Il22*$^{-/-}$ mock n=2, WT mock n=2. The fold increase is expressed as the ratio between the mock-infected WT or *Il-22* mice and infected mice, respectively. Bars represent the geometric mean ±SD. Significant differences are indicated by $p<0.05$ (*). ns = not significant.

Because our results demonstrated that IL-22 is essential for the protection of mice against *C. rodentium* and displayed lower induction of some antimicrobial genes in the gut, we investigated whether specific IL-22-dependent antimicrobial responses contributed to host protection during this infection. Previous studies have shown that IL-22 plays a protective role during *Clostridium difficile* through the regulation of the complement system by augmenting phagocytosis via modulating the expression and

bacterial binding of complement C3 (166). Thus, we decided to perform *C. rodentium* infection in C3-deficient mice to evaluate whether IL-22-mediated responses through C3 would be essential for the protective role of this cytokine in mice. Our results showed that All WT and *C3*$^{-/-}$ mice survived for 25 days after *C. rodentium* infection (Figure 24A), displayed comparable levels of *C. rodentium* colonization, and showed no signs of weight loss (Figures 24B and C). Thus, the absence of C3 does not reduce mouse survival during *C. rodentium* infection.

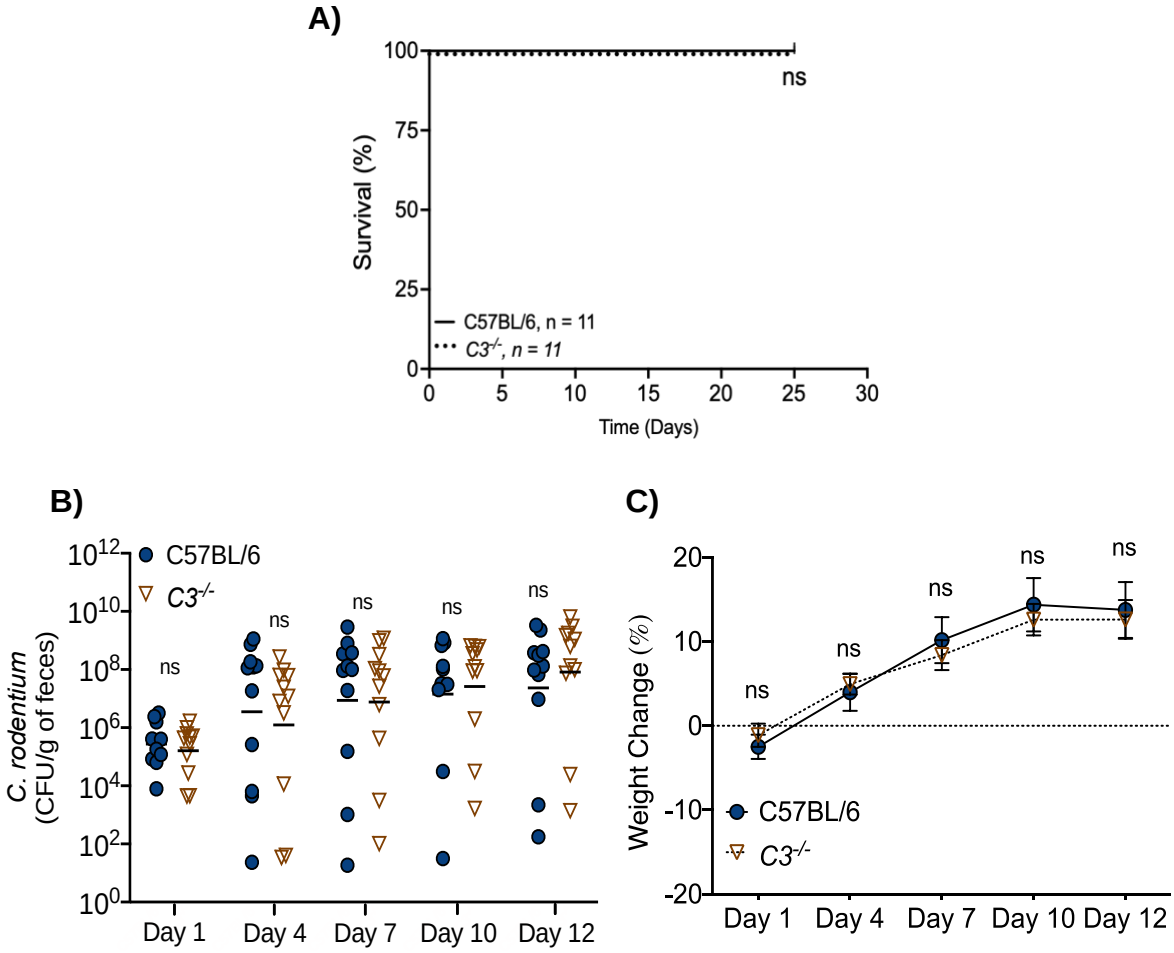

Figure 24. **C3-mediated responses are not critical for defense against *C. rodentium* infection. (A)** Survival course of WT and *C3*$^{-/-}$ mice infected with *C. rodentium*. **(B)** Fecal CFU of infected mice at days 1, 4, 7, 10 and 12 post-infection. **(C)** Weight course of mice infected with *C. rodentium*. Each symbol represents an individual mouse. Bars represent the geometric mean ± SD. Non-significant differences are indicated by ns.

One critical response under IL-22 regulation is the induction of Lipocalin-2. Lipocalin-2 is an antimicrobial protein known to inhibit bacterial growth by sequestering the siderophore enterobactin (167,183). However, some bacterial pathogens, including *Salmonella enterica* serovar Typhimurium, can acquire iron through alternative mechanisms and are, therefore, resistant to lipocalin-2 (41,43). Since IL-22 induces lipocalin-2 expression by gut epithelial cells (41), we examined whether lipocalin-2 confers protection during *C. rodentium* infection. To investigate the contribution of Lipocalin-2 during *C. rodentium* infection, we challenged Lipocalin-2 deficient mice to track survival, *C. rodentium* colonization, and weight loss upon infection. Only one *Lcn2*$^{-/-}$ mouse succumbed to infection compared to WT mice (Figure 25A) within the following 25 days post-infection. However, we did not observe differences in the colonization levels of mice (Figures 25B and C). Thus, lipocalin-2 deficiency does not recapitulate the survival defect seen in *Il22*$^{-/-}$ mice during *C. rodentium* infection.

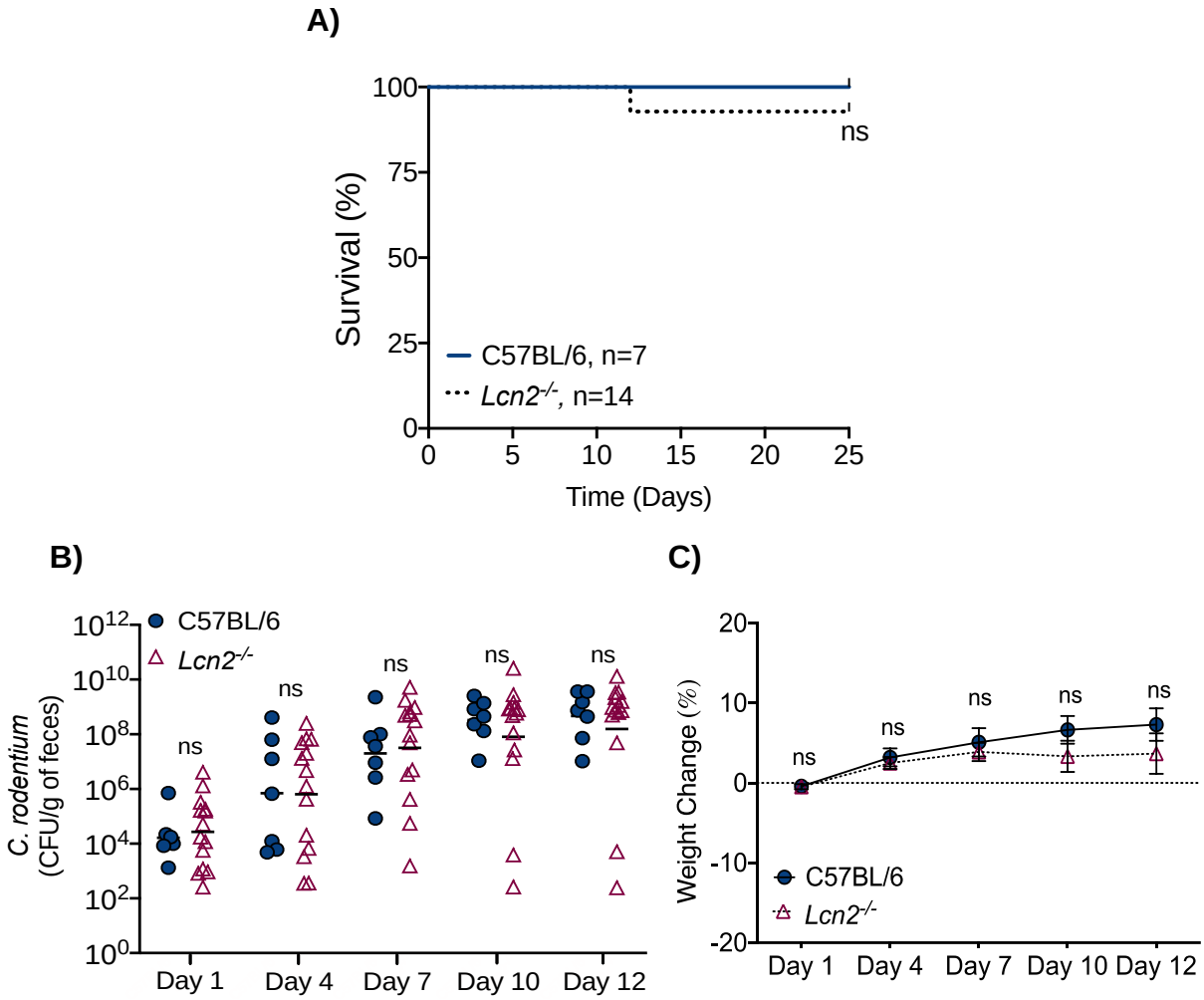

Figure 25. Lipocalin-2 is not the primary mechanism responsible for IL-22-regulated protection against *C. rodentium* infection. (A) Survival course of WT and *Lcn2*$^{-/-}$ mice infected with *C. rodentium*. **(B)** Fecal CFU of infected mice at days 1, 4, 7, 10 and 12 post-infection. **(C)** Weight course of mice infected with *C. rodentium*. Each symbol represents an individual mouse. Bars represent the geometric mean ± SD. Non-significant differences are indicated by ns.

Besides Lipocalin-2, colonic epithelial cells stimulated with IL-22 also express the antimicrobial protein calprotectin (41), a heterodimer composed of S100A8 and S100A9, which is also highly expressed by neutrophils (45). Calprotectin exerts its antimicrobial activity by restricting metal nutrient availability to pathogens (45,143). While some bacteria are susceptible to the antimicrobial activity of calprotectin (141,184), others are resistant (185,186). Thus, we next investigate whether calprotectin contributes to

protection against *C. rodentium* infection. We inoculated *S100a9*$^{-/-}$ mice with *C. rodentium,* followed by monitoring mouse survival and colonization up to 25 days post-infection. The *S100a9*$^{-/-}$ mice did not show differences in any of the parameters as compared to the WT mice (Figures 26A, B, and C). Therefore, *C. rodentium* is resistant to calprotectin, and that *S100a9*$^{-/-}$ mice do not phenocopy the survival impairment observed in *Il22*$^{-/-}$ mice.

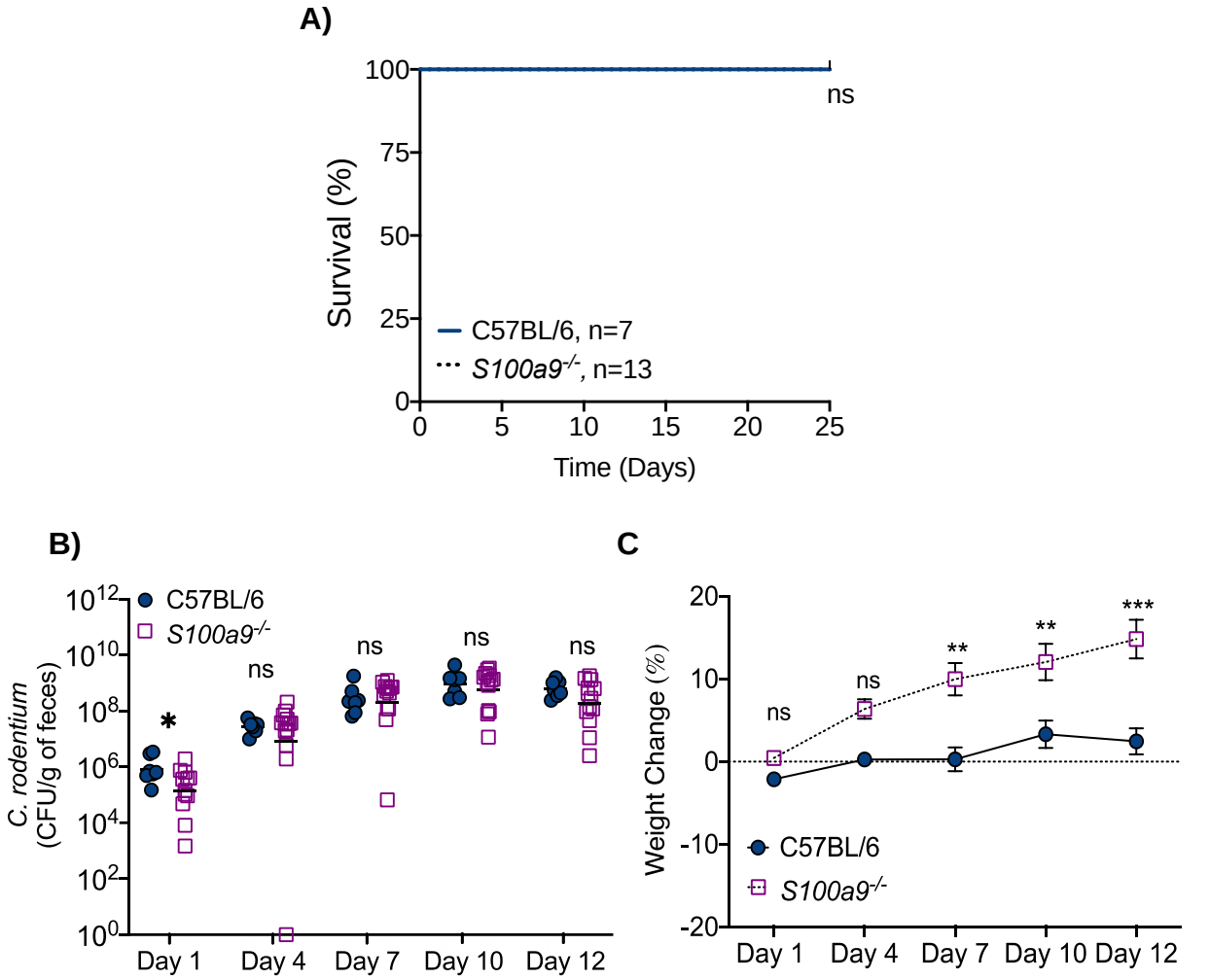

Figure 26. Calprotectin is not essential for host protection during *C. rodentium* infection. (A) Survival course of WT and *s1009*$^{-/-}$ mice infected with *C. rodentium*. **(B)** Fecal CFU of infected mice at days 1, 4, 7, 10 and 12 post-infection. **(C)** Weight course of mice infected with *C. rodentium*. Each symbol represents an individual mouse. Bars represent the geometric mean ± SD. Significant differences are indicated by p<0.01 (**) and p<0.01 (***). Non-significant differences are indicated by ns.

REG3γ and REG3β are two antimicrobial lectins secreted by Paneth cells and epithelial cells. They function by directly binding to bacterial membranes and are upregulated in response to IL-22 (39,42,47,163,187,188). To assess the specific contribution of endogenous Reg3γ and Regβ during *C.rodentium* infection, we infected *Reg3*$\beta^{-/-}$ and *Reg3*$\gamma^{-/-}$ mice, monitoring colonization levels, weight loss, and survival. Neither REG3γ nor REG3β are critical for host protection against *C. rodentium*, since the knockout mice did not exhibit increased susceptibility to *C. rodentium* infection, and only one mouse for *Reg3*$\gamma^{-/-}$ died in 25 days post-infection (Figure 27A, Figure 28A). *C. rodentium* colonization of *Reg3y*$^{-/-}$ and *Reg3*$\beta^{-/-}$ mice were similar compared with WT mice (Figure 27B, Figure 28B), and no significant difference in weight loss in the knockout groups was detected (Figure 27C, Figure 28C). Thus, our data suggest that the antimicrobial properties of REG3γ or REG3β are not essential for the survival of mice during *C. rodentium* infection.

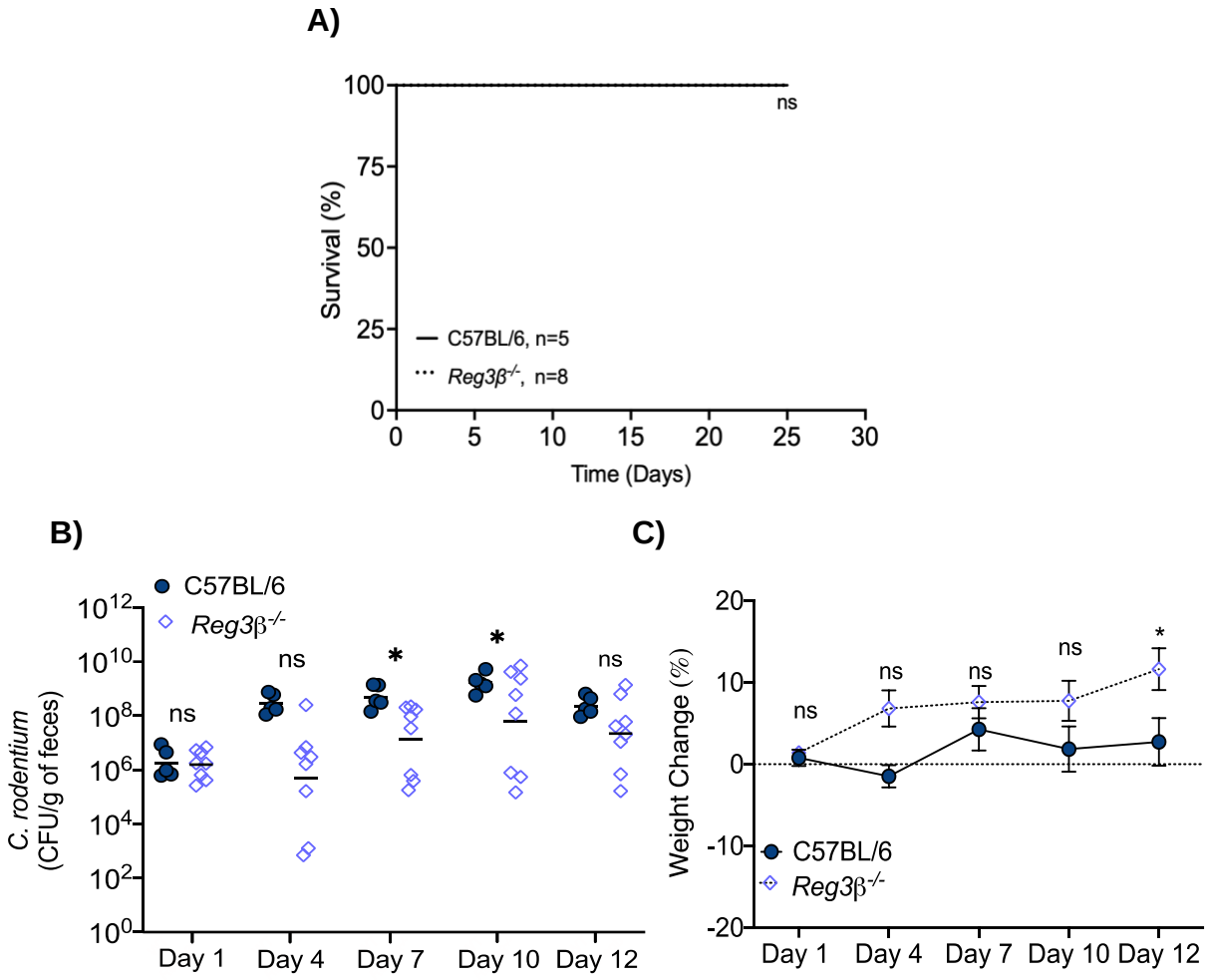

Figure 27. REG3β is not critical for host protection against *C. rodentium* infection. (A) Survival course of WT and REG3β$^{-/-}$ mice infected with *C. rodentium*. **(B)** Fecal CFU of infected mice at days 1, 4, 7, 10 and 12 post-infection. **(C)** Weight course of mice infected with *C. rodentium*. Each symbol represents an individual mouse. Bars represent the geometric mean ± SD. Significant differences are indicated by $p<0.05$ (*). Non-significant differences are indicated by ns.

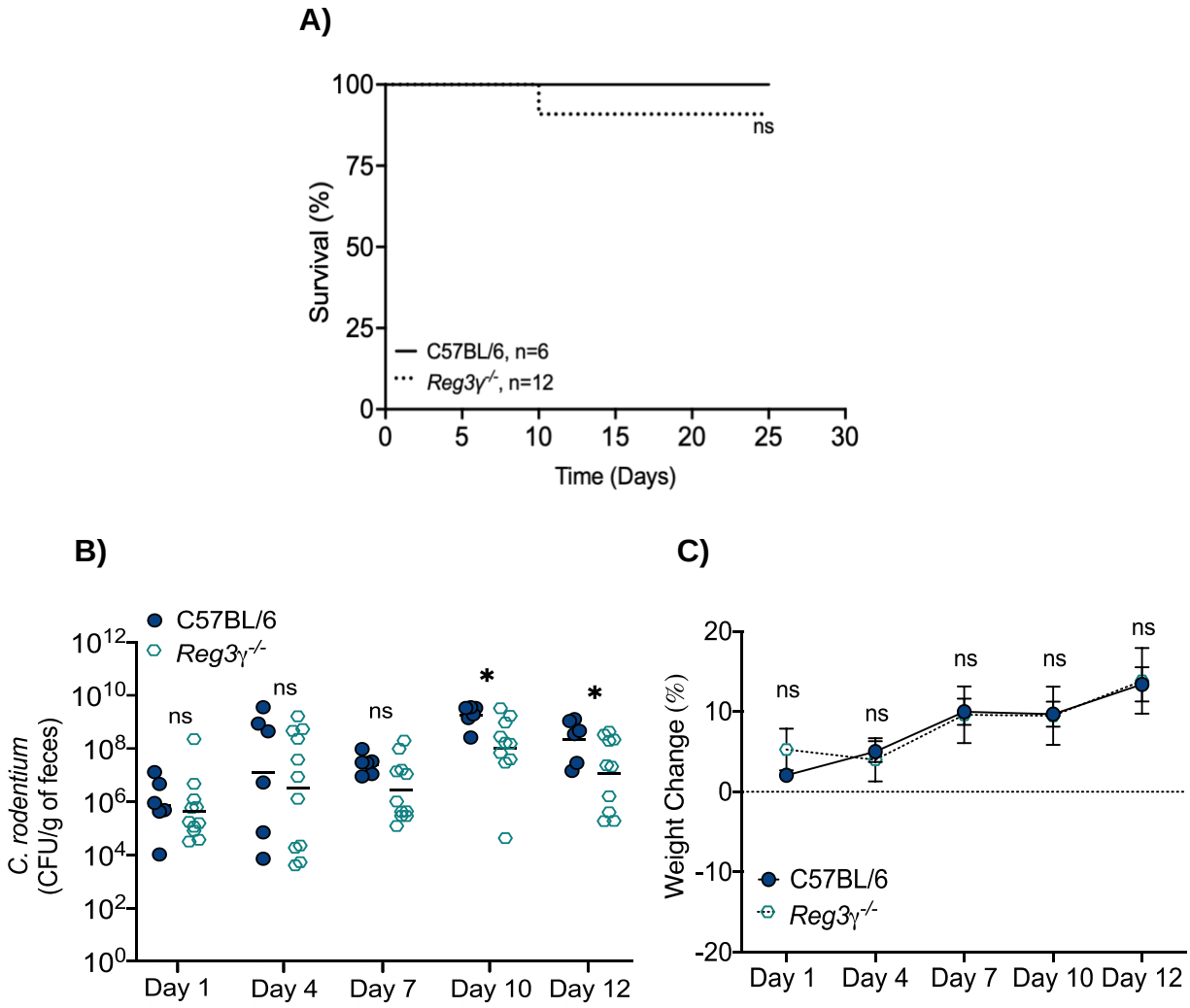

Figure 28. REG3γ is not critical for host protection against *C. rodentium* infection. (A) Survival course of WT and *Reg3γ*$^{-/-}$ mice infected with *C. rodentium*. **(B)** Fecal CFU of infected mice at days 1, 4, 7, 10 and 12 post-infection. **(C)** Weight course of mice infected with *C. rodentium*. Each symbol represents an individual mouse. Bars represent the geometric mean ± SD. Significant differences are indicated by $p<0.05$ (*). Non-significant differences are indicated by ns.

Previous studies have shown that mice with a deficiency in IL-22 signaling exhibit a compromised integrity of the colon and an impaired barrier function of the gut (39). Our results indicate that the severity of disease observed in *Il22*$^{-/-}$ mice infected with *C. rodentium* cannot be replicated by mice lacking individual antimicrobial responses modulated by IL-22. To explore alternative mechanisms of IL-22-mediated protection, we compared the inflammatory response in the colon of WT mice and *Il22*$^{-/-}$ mice 12 days

after *C. rodentium* infection, which is the time point at which mice began to succumb in our colony. Upon gross examination, the colon lengths of WT mice were similar to the uninfected mice, whereas IL-22-deficient mice displayed inflamed colon with significant colonic shortening in comparison to WT mice and uninfected mice. In contrast, infected *Il22*$^{-/-}$ mice had significantly shorter colons, indicative of inflammation (Figure 29A). To further assess the degree of inflammation between the groups, we collected entire colons and prepared Swiss rolls for histopathological analysis. The total colitis score confirmed considerably higher inflammation in *Il22*$^{-/-}$ mice compared to WT mice (Figure 29B). This increased inflammation was characterized by enhanced epithelial injury and a substantial increase in infiltrated mononuclear cells and neutrophilic crypt abscesses (Figures 29C and 29D).

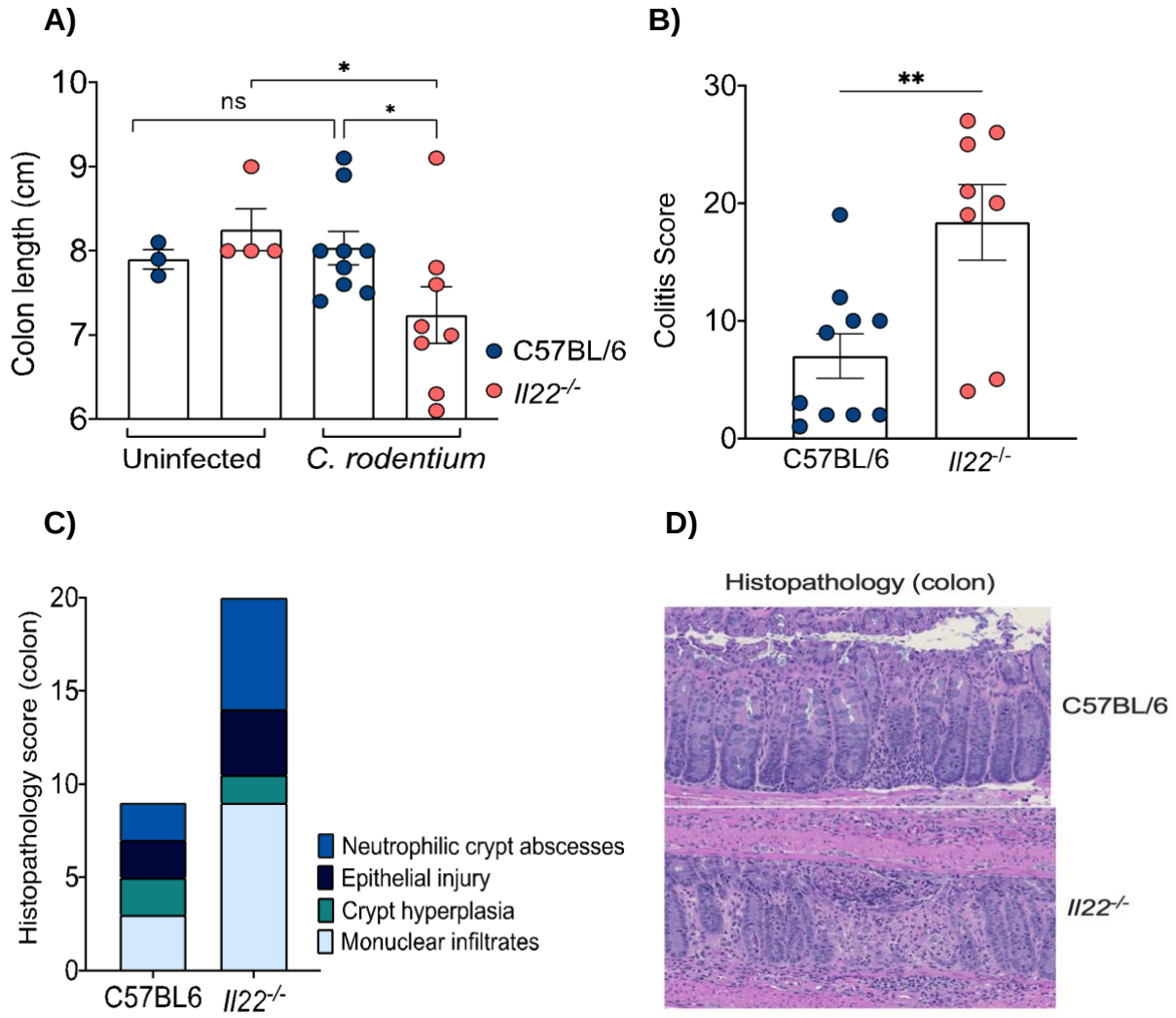

Figure 29. L-22 is essential to ameliorate *C. rodentium*-induced inflammation in the recovering phase. (A) Colon length of infected and uninfected WT and *Il-22*$^{-/-}$ mice at day 12 post-infection**. (B)** Colitis scores of infected WT and *Il22*$^{-/-}$ mice at day 12 post-infection. (**A, B**) Each circle represents an individual mouse **(C)** Histopathology scores with individual subcategories at day 12 post-infection **(D)** Representative H&E stained colon sections from mice infected with *C. rodentium*. Each circle represents an individual mouse. Bars represent the geometric mean ±SD. Significant differences are indicated by p<0.05 (*), p<0.01 (**), and p<0.01 (***). ns = not significant.

Since our data suggest that epithelial injury was higher in *Il22*$^{-/-}$ mice, we decided to analyze genes involved in inflammation responses. The expression levels of *Il1b*, *Il6*, *Nos2*, and *Ifng* (encoding IL-1β, IL-6, iNOS, and IFN-γ) were similarly increased in both WT and *Il22*$^{-/-}$ mice at this time point (Figure 30). Besides, the higher colonic inflammation observed in *Il22*$^{-/-}$ mice corresponded with reduced induction of the *Tnfa, Il17a,* and *Il10*

genes (encoding TNF-α, IL-17A, and the anti-inflammatory cytokine IL-10). Together, these results indicate that IL-22 ameliorates colonic inflammation triggered by *C. rodentium* infection.

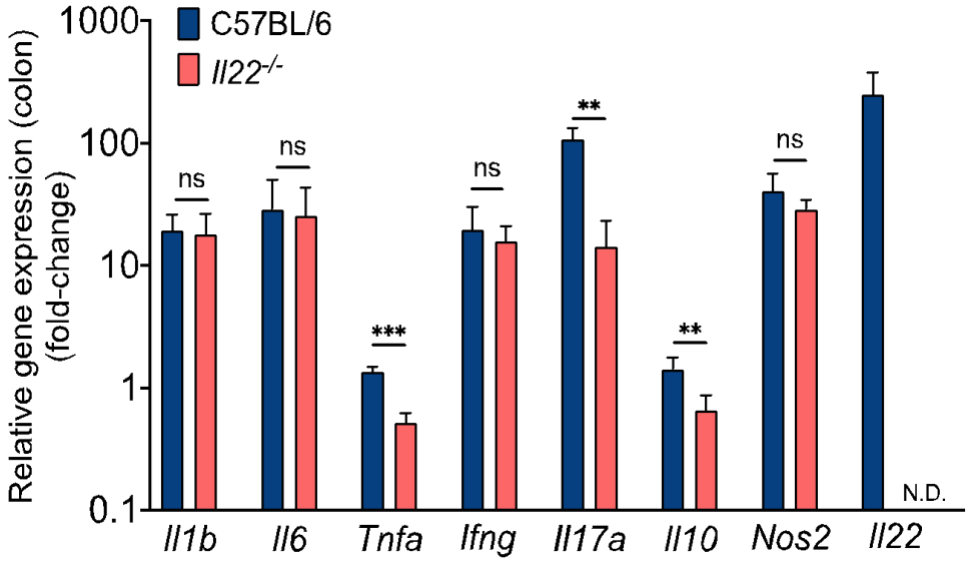

Figure 30. Analysis of cytokine gene expression from infected *Il22*$^{-/-}$ mice. A real-time polymerase chain reaction (RT-qPCR) was used to determine the expression of *Il1b*, *Il6*, *Tnfa, Ifng*, *Il17a, Il10, Nos2,* and *Il22* in the colon of WT and *Il22*$^{-/-}$ mice 12 days after infection. WT n=4, *Il22*$^{-/-}$ n=4 infected mice, *Il22*$^{-/-}$ mock n=2, WT mock n=2. Bars represent the geometric mean ±SD. Significant differences are indicated by $p<0.05$ (*), $p<0.01$ (**), and $p<0.01$ (***). ns = not significant.

Studies have shown the involvement of IL-22 in enhancing the integrity and barrier function of the gut epithelium (39,189). In light of this, we investigated whether the expression of genes known to be associated with gut integrity and permeability is altered in infected *Il22*$^{-/-}$ mice. Our findings demonstrated that *Il22*$^{-/-}$ mice exhibited reduced expression of the integrin beta-1 gene (*Itgb1*), which is known for intestinal cell function and proliferation (84,190,191) when compared to WT mice (Figure 31). Furthermore, *Il22*$^{-/-}$ mice have diminished expression of genes encoding occludin-1, claudin-2, and cadherin-1, all of which are involved in tight junctions and adherence junctions in the intestinal epithelium (174,192–194) (Figure 31). Besides, Fucosyltransferase-2 and

mucin-2 have important roles in intestinal epithelial function and have been associated with host resistance against *C. rodentium* (175,195,196). In our study, although *Muc2* expression did not show a significant difference between infected WT and *Il22*$^{-/-}$ mice, there was a noticeable trend toward a decrease in *Il22*$^{-/-}$ mice. Conversely, *Fut2* expression was significantly lower in infected *Il22*$^{-/-}$ mice. Together, our data suggest that IL-22 is essential for the host responses involved in controlling epithelial injury and inflammation during *C. rodentium* infection.

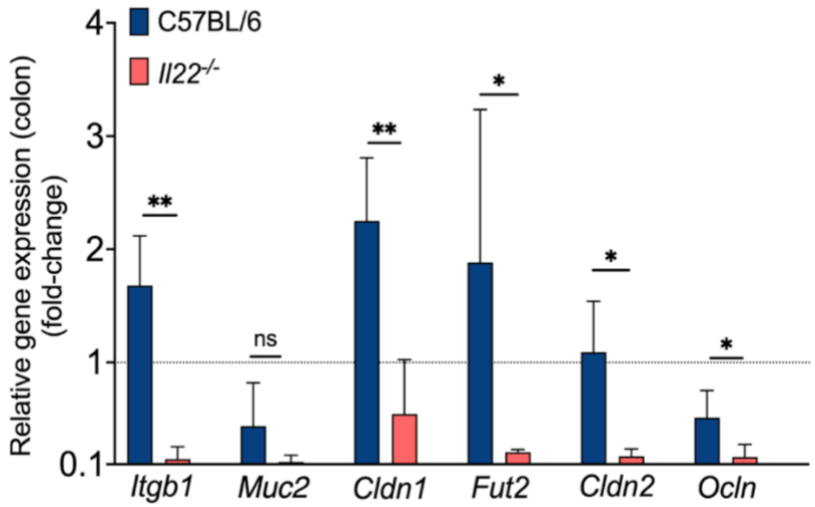

Figure 31. Expression of genes involved in gut permeability from infected *Il22*$^{-/-}$ mice. A real-time polymerase chain reaction (RT-qPCR) was used to determine the expression of *Itgb1*, *Muc2*, *Cldn1*, *Fut2*, *Cldn2,* and *Ocln* in the colon of WT and *Il-22*$^{-/-}$ mice 12 days after infection. WT infected n=4, *Il22*$^{-/-}$ infected n=4, *Il22*$^{-/-}$ mock n=2, WT mock n=2. Bars represent the geometric mean ± SD. Significant differences are indicated by $p<0.05$ (*), $p<0.01$ (**), and ns = not significant.

To assess whether gut permeability is functionally compromised in *Il22*$^{-/-}$ mice, we conducted a FITC-dextran permeability assay. 12 days after *C. rodentium* infection, WT and *Il22*$^{-/-}$ mice were infected with *C. rodentium,* and FITC-dextran was orally administered to mice. Four hours later, plasma was collected from animals, and FITC fluorescence was determined. In WT mice infected with *C. rodentium*, levels of FITC-

dextran detected in the plasma were similar to those in uninfected mice, suggesting that the gut barrier was intact at this time point. In contrast, FITC-dextran levels of *C. rodentium*-infected *Il22*$^{-/-}$ mice were significantly higher compared to non-infected mice and infected WT mice (Figure 32A), confirming that IL-22 is essential for gut epithelial integrity. Moreover, the CFU burden in the spleen of *Il22*$^{-/-}$ mice was significantly higher than in WT mice, thus corroborating the increased permeability observed in *Il22*$^{-/-}$ mice (Figure 32B). Additionally, at this time point, we recovered significantly higher *C. rodentium* CFU from the spleen of *Il22*$^{-/-}$ mice compared to WT mice, providing further evidence of the increased permeability observed in *Il22*$^{-/-}$ mice (Figure 32B). Together, our data indicate that IL-22 plays a crucial role in maintaining barrier function and the integrity of intestinal epithelial cells during *C. rodentium* infection. The “leaky gut” phenomenon and increased pathogen dissemination likely represent key mechanisms through which IL-22 deficiency results in increased susceptibility to *C. rodentium* of C57BL/6 mice.

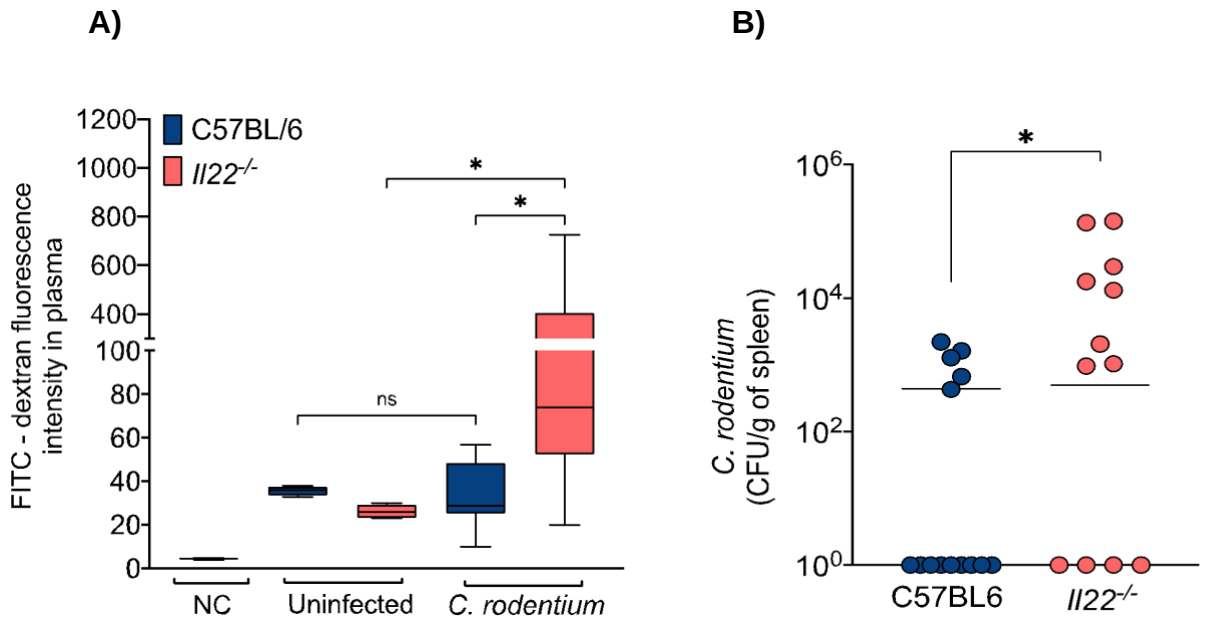

Figure 32. Mice lacking IL-22 responses display gut permeability alterations and increased bacterial translocation to the spleen during *C. rodentium* infection. (A) Intestinal permeability of *Il22*$^{-/-}$ mice was determined by FITC-dextran assayed in the plasma of mice infected with *C. rodentium* at day 12 post-infection. Uninfected WT and *Il22*$^{-/-}$ mice were used as baseline controls for intestinal permeability. Infected WT (n=10), infected *Il-22*$^{-/-}$ (n=7), *Il22*$^{-/-}$ mock (n=2), WT mock (n=4). Each box represents the median, and whiskers represent the minimum and maximum of FITC-dextran concentration in plasma (**B)** *C. rodentium* CFU in the spleen 12 days post-infection. Bars represent the geometric mean. Each circle represents an individual mouse. Significant differences are indicated by $p<0.05$ (*) and ns = not significant.

3.4. Discussion

IL-22 is a cytokine that promotes host defense through various mechanisms, including the induction of antimicrobial responses and enhancement of the intestinal mucosal barrier (197). During *C. rodentium* infection, IL-22 is essential for pathogen clearance and disease amelioration (39,118,175,198). Indeed, *Il22*$^{-/-}$ mice exhibit impaired host defense against *C. rodentium* infection, and this effect is independent of adaptive immunity (39).

Our findings align with previous studies demonstrating that IL-22 is essential for mice to survive *C. rodentium* infection (39,118,175,198). In our study, *Il22*$^{-/-}$ mice exhibited higher levels of *C. rodentium* colonization and increased colonic inflammation

(Figures 1A, 5B, and 5C). At 12 days post-infection, *Il22*$^{-/-}$ mice began to succumb to the infection, whereas WT mice started to recover and cleared *C. rodentium* from the gut. By day 25, almost all infected *Il22*$^{-/-}$ mice died, while all infected WT mice survived (Figure 1). *C. rodentium* infection in mice with a mild disease phenotype, such as C57BL/6 mice, can be divided into four distinct phases (97): The establishment phase (1-3 days post-infection), the expansion phase (4 - 8 days post-infection), steady-state phase (8-12 days post-infection), and the clearance phase (typically occurring after 12 days post-infection), in which the host begins to recover from the infection and clears the pathogen until no CFU can be detected (97). In the absence of IL-22, C57BL/6 mice fail to transition into the clearance phase. Instead of effectively clearing the infection, these mice succumb to the disease.

Even though the pivotal role of IL-22 for host protection against *C. rodentium* infection has been established, our understanding of whether specific antimicrobial responses downstream of IL-22 contribute to this phenotype remains limited. During infection, many pathogens require the acquisition of metal nutrients to successfully colonize the host (43,199–202). Mice lacking IL-22 exhibit decreased expression of the heme-binding protein hemopexin. Thus, *C. rodentium* can more effectively acquire heme iron, leading to greater dissemination to extraintestinal sites in *Il22*$^{-/-}$ mice (118). During *C. rodentium* infection, the two metal-sequestering antimicrobial proteins lipocalin-2 and calprotectin are highly expressed in the colon. Nevertheless, we found that mice lacking either of these proteins do not exhibit increased susceptibility to *C. rodentium* infection. In other words, a single deficiency of lipocalin-2 or calprotectin does not recapitulate the phenotype observed in *Il22*$^{-/-}$ mice and further suggests that, *in vivo*, *C. rodentium* is resistant to metal sequestration by lipocalin-2 and calprotectin.

The complement system is an important host response that eliminates pathogens by promoting their recognition, ingestion, and destruction by phagocyte cells (203). IL-22 has been shown to control infections by inducing the complement system, resulting in enhanced bacterial phagocytosis through the modulation of complement C3 expression (166). In our study, we found that adult *C3*$^{-/-}$ mice exhibited resistance to *C. rodentium* infection similar to that of WT mice. A separate study reported that young (21-day-old) *C3*$^{-/-}$ mice were found to be more susceptible to *C. rodentium* infection than age-matched WT mice (204). Thus, although C3 deficiency does not lead to lethal *C. rodentium* infection in adult mice, the role of C3 may be more critical in early life.

IL-22 regulates the expression of C-type lectins such as Reg3β and Reg3γ (39,163). Members of the Reg3 protein family exert direct antibacterial activity by binding to components of bacterial membranes, including peptidoglycan (163,205) and lipid A (47) which assist the host in controlling infections (206,207). During *C. rodentium* infection, the endogenous intraperitoneal administration of Reg3γ was sufficient to partially rescue the mortality of *Il22*$^{-/-}$ mice (208). However, another study showed that *Reg3γ*$^{-/-}$ mice exhibited resistance similar to that of WT mice during *C. rodentium* infection (175). Our findings are in alignment with this study, as neither *Reg3β* $^{-/-}$ nor *Reg3γ*$^{-/-}$ mice in our colony succumb to *C. rodentium* disease. These collective observations suggest that the antimicrobial activity of Reg3 family proteins in the gut may not be the primary mechanism by which IL-22 provides its protection against *C. rodentium* infection.

In addition to inducing several antimicrobial responses, IL-22 modulates barrier function, thereby ameliorating colonic inflammation (189,198,209). Consistent with these reports, we found that *Il22*$^{-/-}$ mice exhibited more severe inflammation than WT mice following *C. rodentium* infection. Although several proinflammatory cytokine genes,

including *Il1b*, *Il6*, and *Ifng*, were similarly induced in both *Il22*$^{-/-}$ and WT mice, reduced expression of other cytokines (*Il17a, Tnfa,* and *Il10*) may help explain the increased pathology observed in *Il22*$^{-/-}$ mice. Moreover, the higher colonic inflammation in *Il22*$^{-/-}$ mice could potentially be attributed to an inadequate epithelial repair mechanism. Key genes responsible for maintaining epithelial integrity and promoting recovery, such as those encoding claudin-2, occludin, e-cadherin, and β1-integrin, displayed reduced expression during infection in *Il22*$^{-/-}$ mice. Functionally, disruption of the epithelial barrier in *Il22*$^{-/-}$ mice was confirmed by elevated FITC levels in the serum and increased *C. rodentium* translocation to the spleen. These findings are consistent with previous studies that emphasize the role of IL-22 in promoting and maintaining gut epithelial integrity (175,189,198,209). Notably, mice lacking claudin-2 were found to be highly susceptible to *C. rodentium* infection (174), albeit not to the level of *Il22*$^{-/-}$ mice.

Another gene with reduced expression in infected *Il22*$^{-/-}$ mice is *fut2*, which encodes a 1,2-fucosyltransferase involved in epithelial glycosylation (210). The IL-22 receptor IL-22RA1 has been shown to promote intestinal fucosylation, thereby enhancing colonization resistance by increasing anaerobic commensal diversity. This, in turn, prevents the translocation of opportunist pathogens during inflammation and infection, ultimately supporting host recovery (175). The study of Pickard et al. further supports these observations, demonstrating that fucose is liberated and metabolized by the gut microbiota in response to microbial stimuli. This process fosters the integration of host and gut microbes, providing protection by decreasing the expression of bacterial virulence genes and enhancing host tolerance to *C. rodentium* (211). Taken together, these studies underscore the important role of IL-22 and Fut2 in maintaining proper fucosylation levels to enhance host defense against pathogens such as *C. rodentium*. Thus, the observed

susceptibility to *C. rodentium* infection in *Il22*$^{-/-}$ mice may also be attributed, at least in part, to the diminished expression of *Fut2*.

Our findings collectively suggest that the heightened susceptibility of *Il22*$^{-/-}$ mice to *C. rodentium* infection can be attributed to a combination of redundant mechanisms. Our study expands on and reinforces the essential role of IL-22 for the resolution of *C. rodentium* infection, which is not solely dependent on the IL-22-induced antimicrobial responses calprotectin, lipocalin-2, Reg3β, Reg3γ, and C3. Additionally, our research broadens our understanding of the role of IL-22 in maintaining and repairing the integrity of the gut epithelial barrier in the recovery phase of infection. This process emerges as necessary for the host's ability to recover from *C. rodentium* infection.

Chapter IV: Conclusion

Our data demonstrated that through the QseC-mediated signaling, *C. rodentium* has an increased ability to survive neutrophil-mediated immune responses, thereby facilitating systemic spreading *in vivo*. Besides, the QseC sensor is involved in modulating *C. rodentium's* O-antigen chain of LPS, which could be involved in its resistance to neutrophil killing. In addition, mucosal immune mechanisms during *C. rodentium* infections are complex; in our study, CXCR2-mediated neutrophil responses are essential to protect the host from *C. rodentium* colonization, inflammation, and systemic spreading. Moreover, QseC is directly involved in triggering *C. rodentium*-mediated intestinal inflammation and inducing transmissible colonic crypt hyperplasia, the hallmark pathological feature of *C. rodentium* infection. Overall, our *in vivo* and *in vitro* data confirms that the QseC sensor is essential for *C. rodentium* virulence, and it is involved in *C. rodentium's* ability to efficiently evade immune response and establish the disease.

Besides, IL-22 is critical for the resolution of *C. rodentium* infection, which does not seem to be solely dependent on its downstream antimicrobial peptides calprotectin, REG3, Lipocalin-2, and C3, but could also be attributed to IL-22 epithelial mucosal repair mechanisms. These findings broaden our understanding of the role of IL-22 in protecting the host during *C. rodentium* infection.

In summary, our study demonstrated that *C. rodentium* uses the QseC sensor to establish the infection and evade the host response, which involves IL-22-mediated protection for the host to efficiently recover from the infection and clean the disease.

REFERENCES

1. Greenwood-Van Meerveld B, Johnson AC, Grundy D. Gastrointestinal Physiology and Function. In: Greenwood-Van Meerveld B, editor. Gastrointestinal Pharmacology [Internet]. Cham: Springer International Publishing; 2017 [cited 2023 Sep 19]. p. 1–16. (Handbook of Experimental Pharmacology). Available from: https://doi.org/10.1007/164_2016_118

2. Fedoruk MJ, Guidotti TL. Gastrointestinal System. In: Wexler P, editor. Encyclopedia of Toxicology (Second Edition) [Internet]. New York: Elsevier; 2005 [cited 2022 Sep 20]. p. 410–6. Available from: https://www.sciencedirect.com/science/article/pii/B0123694000004464

3. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017 Jun 1;474(11):1823–36.

4. Hou K, Wu ZX, Chen XY, Wang JQ, Zhang D, Xiao C, et al. Microbiota in health and diseases. Signal Transduct Target Ther. 2022 Apr 23;7(1):1–28.

5. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol. 2021 Jan;19(1):55–71.

6. Belkaid Y, Hand T. Role of the Microbiota in Immunity and inflammation. Cell. 2014 Mar 27;157(1):121–41.

7. Gieryńska M, Szulc-Dąbrowska L, Struzik J, Mielcarska MB, Gregorczyk-Zboroch KP. Integrity of the Intestinal Barrier: The Involvement of Epithelial Cells and Microbiota—A Mutual Relationship. Anim Open Access J MDPI. 2022 Jan 8;12(2):145.

8. Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. Nat Immunol. 2013 Jul;14(7):646–53.

9. Perez-Lopez A, Behnsen J, Nuccio SP, Raffatellu M. Mucosal immunity to pathogenic intestinal bacteria. Nat Rev Immunol. 2016 Mar;16(3):135–48.

10. Cornick S, Tawiah A, Chadee K. Roles and regulation of the mucus barrier in the gut. Tissue Barriers. 2015 Jan 15;3(1–2):e982426.

11. Kim JM. Antimicrobial Proteins in Intestine and Inflammatory Bowel Diseases. Intest Res. 2014 Jan;12(1):20–33.

12. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol. 2010 Mar;10(3):159–69.

13. Soderholm AT, Pedicord VA. Intestinal epithelial cells: at the interface of the microbiota and mucosal immunity. Immunology. 2019 Dec;158(4):267–80.

14. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009 Nov;9(11):799–809.

15. Murch S. 5 - Gastrointestinal Mucosal Immunology and Mechanisms of Inflammation. In: Wyllie R, Hyams JS, Kay M, editors. Pediatric Gastrointestinal and Liver Disease (Sixth Edition) [Internet]. Philadelphia: Elsevier; 2021 [cited 2022 Oct 15]. p. 40-52.e3. Available from: https://www.sciencedirect.com/science/article/pii/B9780323672931000050

16. McGhee JR, Fujihashi K. Inside the Mucosal Immune System. PLOS Biol. 2012 Sep 25;10(9):e1001397.

17. Mittal R, Debs LH, Patel AP, Nguyen D, Patel K, O'Connor G, et al. Neurotransmitters: The critical modulators regulating gut-brain axis. J Cell Physiol. 2017 Sep;232(9):2359–72.

18. Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol. 2001 Nov;2(11):1004–9.

19. Hugenholtz F, de Vos WM. Mouse models for human intestinal microbiota research: a critical evaluation. Cell Mol Life Sci. 2018 Jan 1;75(1):149–60.

20. Vivier E, Malissen B. Innate and adaptive immunity: specificities and signaling hierarchies revisited. Nat Immunol. 2005 Jan;6(1):17–21.

21. Mörbe UM, Jørgensen PB, Fenton TM, von Burg N, Riis LB, Spencer J, et al. Human gut-associated lymphoid tissues (GALT); diversity, structure, and function. Mucosal Immunol. 2021 Jul;14(4):793–802.

22. Akira S, Uematsu S, Takeuchi O. Pathogen Recognition and Innate Immunity. Cell. 2006 Feb 24;124(4):783–801.

23. Ye W, Murata Y. Microbe Associated Molecular Pattern Signaling in Guard Cells. Front Plant Sci [Internet]. 2016 [cited 2023 Oct 3];7. Available from: https://www.frontiersin.org/articles/10.3389/fpls.2016.00583

24. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol. 2009;60:379–406.

25. Yuan Q, Walker WA. Innate Immunity of the Gut: Mucosal Defense in Health and Disease. J Pediatr Gastroenterol Nutr. 2004 May;38(5):463–73.

26. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol. 2005 Dec;6(12):1182–90.

27. Martín-Fontecha A, Lanzavecchia A, Sallusto F. Dendritic Cell Migration to Peripheral Lymph Nodes. In: Lombardi G, Riffo-Vasquez Y, editors. Dendritic Cells [Internet]. Berlin, Heidelberg: Springer; 2009 [cited 2022 Oct 18]. p. 31–49. (Handbook of Experimental Pharmacology). Available from: https://doi.org/10.1007/978-3-540-71029-5_2

28. Krummel MF, Bartumeus F, Gérard A. T cell migration, search strategies and mechanisms. Nat Rev Immunol. 2016 Mar;16(3):193–201.

29. Loo TT, Gao Y, Lazarevic V. Transcriptional regulation of CD4+ TH cells that mediate tissue inflammation. J Leukoc Biol. 2018 Dec;104(6):1069–85.

30. Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^+$T cells: differentiation and functions. Clin Dev Immunol. 2012;2012:925135.

31. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a TH17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature. 2007 Feb;445(7128):648–51.

32. Valle-Noguera A, Ochoa-Ramos A, Gomez-Sánchez MJ, Cruz-Adalia A. Type 3 Innate Lymphoid Cells as Regulators of the Host-Pathogen Interaction. Front Immunol. 2021 Sep 29;12:748851.

33. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JKM, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature. 2009 Feb 5;457(7230):722–5.

34. Bando JK, Colonna M. Innate lymphoid cell function in the context of adaptive immunity. Nat Immunol. 2016 Jun 21;17(7):783–9.

35. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol. 2009 Aug;10(8):864–71.

36. Kreymborg K, Etzensperger R, Dumoutier L, Haak S, Rebollo A, Buch T, et al. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. J Immunol Baltim Md 1950. 2007 Dec 15;179(12):8098–104.

37. Zhang K, Chen L, Zhu C, Zhang M, Liang C. Current Knowledge of Th22 Cell and IL-22 Functions in Infectious Diseases. Pathogens. 2023 Feb;12(2):176.

38. Keir ME, Yi T, Lu TT, Ghilardi N. The role of IL-22 in intestinal health and disease. J Exp Med. 2020 Feb 13;217(3):e20192195.

39. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med. 2008 Mar;14(3):282–9.

40. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M,

et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med. 2006 Oct 2;203(10):2271–9.

41. Raffatellu M, George MD, Akiyama Y, Hornsby MJ, Nuccio SP, Paixao TA, et al. Lipocalin-2 Resistance Confers an Advantage to *Salmonella enterica* Serotype Typhimurium for Growth and Survival in the Inflamed Intestine. Cell Host Microbe. 2009 May 21;5(5):476–86.

42. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The Antibacterial Lectin RegIIIγ Promotes the Spatial Segregation of Microbiota and Host in the Intestine. Science. 2011 Oct 14;334(6053):255–8.

43. Gerner RR, Nuccio SP, Raffatellu M. Iron at the host-microbe interface. Mol Aspects Med. 2020 Oct 1;75:100895.

44. Berger T, Togawa A, Duncan GS, Elia AJ, You-Ten A, Wakeham A, et al. Lipocalin 2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. Proc Natl Acad Sci U S A. 2006 Feb 7;103(6):1834–9.

45. Corbin BD, Seeley EH, Raab A, Feldmann J, Miller MR, Torres VJ, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science. 2008 Feb 15;319(5865):962–5.

46. Mukherjee S, Bassler BL. Bacterial quorum sensing in complex and dynamically changing environments. Nat Rev Microbiol. 2019 Jun;17(6):371–82.

47. Miki T, Holst O, Hardt WD. The Bactericidal Activity of the C-type Lectin RegIIIβ against Gram-negative Bacteria involves Binding to Lipid A. J Biol Chem. 2012 Oct 5;287(41):34844–55.

48. Castro-Sánchez P, Martín-Villa JM. Gut immune system and oral tolerance. Br J Nutr. 2013 Jan;109 Suppl 2:S3-11.

49. Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 2012 Jan 1;3(1):4–14.

50. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity. 2007 May;26(5):579–91.

51. Traxinger BR, Richert-Spuhler LE, Lund JM. Mucosal tissue regulatory T cells are integral in balancing immunity and tolerance at portals of antigen entry. Mucosal Immunol. 2022 May;15(3):398–407.

52. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 2008 Apr;28(4):546–58.

53. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J Exp Med. 2007 Jun 11;204(6):1303–10.

54. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood. 2007 Aug 15;110(4):1225–32.

55. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med. 2007 Aug 6;204(8):1775–85.

56. Chistiakov DA, Bobryshev YV, Kozarov E, Sobenin IA, Orekhov AN. Intestinal mucosal tolerance and impact of gut microbiota to mucosal tolerance. Front Microbiol. 2015 Jan 13;5:781.

57. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Förster R, et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J Exp Med. 2005 Oct 17;202(8):1063–73.

58. Takiishi T, Fenero CIM, Câmara NOS. Intestinal barrier and gut microbiota: Shaping our immune responses throughout life. Tissue Barriers. 2017 Oct 2;5(4):e1373208.

59. Bohnhoff M, Miller CP. Enhanced susceptibility to *Salmonella* infection in streptomycin-treated mice. J Infect Dis. 1962;111:117–27.

60. Collins FM, Carter PB. Growth of salmonellae in orally infected germfree mice. Infect Immun. 1978 Jul;21(1):41–7.

61. Miller CP, Bohnhoff M, Rifkind D. The Effect of an Antibiotic on the Susceptibility of the Mouse's Intestinal Tract to Salmonella Infection. Trans Am Clin Climatol Assoc. 1957;68:51–8.

62. Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, et al. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. Infect Immun. 2008 Oct;76(10):4726–36.

63. Baldwin GC. The biology of granulocyte-macrophage colony-stimulating factor: effects on hematopoietic and nonhematopoietic cells. Dev Biol. 1992 Jun;151(2):352–67.

64. Suratt BT, Petty JM, Young SK, Malcolm KC, Lieber JG, Nick JA, et al. Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. Blood. 2004 Jul 15;104(2):565–71.

65. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. Trends Immunol. 2011 Oct;32(10):452–60.

66. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. Immunology. 2008 Nov;125(3):281–8.

67. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Front Physiol. 2018 Feb 20;9:113.

68. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. J Clin Invest. 2010 Jul;120(7):2423–31.

69. Grespan R, Fukada SY, Lemos HP, Vieira SM, Napimoga MH, Teixeira MM, et al. CXCR2-specific chemokines mediate leukotriene B4-dependent recruitment of

neutrophils to inflamed joints in mice with antigen-induced arthritis. Arthritis Rheum. 2008 Jul;58(7):2030–40.

70. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013 Mar;13(3):159–75.

71. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018 Feb;18(2):134–47.

72. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532–5.

73. Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol. 2006 Apr;8(4):668–76.

74. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med. 2010 Aug 30;207(9):1853–62.

75. Saha P, Yeoh BS, Xiao X, Golonka RM, Singh V, Wang Y, et al. PAD4-dependent NETs generation are indispensable for intestinal clearance of *Citrobacter rodentium*. Mucosal Immunol. 2019 May;12(3):761–71.

76. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016 Aug;14(8):e1002533.

77. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol. 2010 Mar;8(3):207–17.

78. Jang J, Hur HG, Sadowsky M j., Byappanahalli M n., Yan T, Ishii S. Environmental *Escherichia coli*: ecology and public health implications—a review. J Appl Microbiol. 2017;123(3):570–81.

79. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli.* Nat Rev Microbiol. 2004 Feb;2(2):123–40.

80. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev. 2013 Oct;26(4):822–80.

81. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998 Jan;11(1):142–201.

82. Pakbin B, Brück WM, Rossen JWA. Virulence Factors of Enteric Pathogenic *Escherichia coli*: A Review. Int J Mol Sci. 2021 Sep 14;22(18):9922.

83. Fakhouri F, Zuber J, Frémeaux-Bacchi V, Loirat C. Haemolytic uraemic syndrome. Lancet Lond Engl. 2017 Aug 12;390(10095):681–96.

84. Gomes TAT, Elias WP, Scaletsky ICA, Guth BEC, Rodrigues JF, Piazza RMF, et al. Diarrheagenic *Escherichia coli*. Braz J Microbiol Publ Braz Soc Microbiol. 2016 Dec;47 Suppl 1:3–30.

85. Kimmitt PT, Harwood CR, Barer MR. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. Emerg Infect Dis. 2000;6(5):458–65.

86. Mühlen S, Dersch P. Treatment Strategies for Infections With Shiga Toxin-Producing *Escherichia coli*. Front Cell Infect Microbiol. 2020;10:169.

87. Ochoa TJ, Barletta F, Contreras C, Mercado E. New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. Trans R Soc Trop Med Hyg. 2008 Sep;102(9):852–6.

88. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A. 1995 Feb 28;92(5):1664–8.

89. Deng W, Li Y, Vallance BA, Finlay BB. Locus of enterocyte effacement from *Citrobacter rodentium*: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. Infect Immun. 2001 Oct;69(10):6323–35.

90. Franzin FM, Sircili MP. Locus of Enterocyte Effacement: A Pathogenicity Island Involved in the Virulence of Enteropathogenic and Enterohemorragic *Escherichia coli* Subjected to a Complex Network of Gene Regulation. BioMed Res Int. 2015;2015:534738.

91. Deng W, Vallance BA, Li Y, Puente JL, Finlay BB. *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. Mol Microbiol. 2003 Apr;48(1):95–115.

92. Gruenheid S, DeVinney R, Bladt F, Goosney D, Gelkop S, Gish GD, et al. Enteropathogenic *E. coli* Tir binds Nck to initiate actin pedestal formation in host cells. Nat Cell Biol. 2001 Sep;3(9):856–9.

93. Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell. 1997 Nov 14;91(4):511–20.

94. Collins JW, Keeney KM, Crepin VF, Rathinam VAK, Fitzgerald KA, Finlay BB, et al. *Citrobacter rodentium*: infection, inflammation and the microbiota. Nat Rev Microbiol. 2014 Sep;12(9):612–23.

95. Luperchio SA, Schauer DB. Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. Microbes Infect. 2001 Apr;3(4):333–40.

96. Petty NK, Bulgin R, Crepin VF, Cerdeño-Tárraga AM, Schroeder GN, Quail MA, et al. The *Citrobacter rodentium* Genome Sequence Reveals Convergent Evolution with Human Pathogenic Escherichia coli. J Bacteriol. 2010 Jan 15;192(2):525–38.

97. Mullineaux-Sanders C, Sanchez-Garrido J, Hopkins EGD, Shenoy AR, Barry R, Frankel G. *Citrobacter rodentium*–host–microbiota interactions: immunity, bioenergetics and metabolism. Nat Rev Microbiol. 2019 Nov;17(11):701–15.

98. Wiles S, Clare S, Harker J, Huett A, Young D, Dougan G, et al. Organ specificity, colonization and clearance dynamics in vivo following oral challenges with the murine

pathogen *Citrobacter rodentium*. Cell Microbiol. 2004 Oct;6(10):963–72.

99. Schauer DB, Falkow S. The eae gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. Infect Immun. 1993 Nov;61(11):4654–61.

100. Willing BP, Vacharaksa A, Croxen M, Thanachayanont T, Finlay BB. Altering Host Resistance to Infections through Microbial Transplantation. PLOS ONE. 2011 Oct 28;6(10):e26988.

101. Espey MG. Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. Free Radic Biol Med. 2013 Feb 1;55:130–40.

102. Guo P, Zhang K, Ma X, He P. *Clostridium* species as probiotics: potentials and challenges. J Anim Sci Biotechnol. 2020 Feb 20;11(1):24.

103. Litvak Y, Byndloss MX, Bäumler AJ. Colonocyte metabolism shapes the gut microbiota. Science. 2018 Nov 30;362(6418):eaat9076.

104. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 2011 May 4;13(5):517–26.

105. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunol. 2016 Apr 22;5(4):e73.

106. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe. 2015 May 13;17(5):662–71.

107. Berger CN, Crepin VF, Roumeliotis TI, Wright JC, Carson D, Pevsner-Fischer M, et al. *Citrobacter rodentium* Subverts ATP Flux and Cholesterol Homeostasis in Intestinal Epithelial Cells In Vivo. Cell Metab. 2017 Nov 7;26(5):738-752.e6.

108. Barthold SW, Coleman GL, Bhatt PN, Osbaldiston GW, Jonas AM. The etiology of transmissible murine colonic hyperplasia. Lab Anim Sci. 1976 Dec;26(6 Pt 1):889–94.

109. Barthold SW, Coleman GL, Jacoby RO, Livestone EM, Jonas AM. Transmissible murine colonic hyperplasia. Vet Pathol. 1978 Mar;15(2):223–36.

110. Lopez CA, Miller BM, Rivera-Chávez F, Velazquez EM, Byndloss MX, Chávez-Arroyo A, et al. Virulence factors enhance Citrobacter rodentium expansion through aerobic respiration. Science. 2016 Sep 16;353(6305):1249–53.

111. Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem. 2014;83:99–128.

112. Gibson DL, Ma C, Bergstrom KSB, Huang JT, Man C, Vallance BA. MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during *Citrobacter rodentium*-induced colitis. Cell Microbiol. 2008 Mar;10(3):618–31.

113. Zhang JM, An J. Cytokines, Inflammation and Pain. Int Anesthesiol Clin. 2007;45(2):27–37.

114. Spehlmann ME, Dann SM, Hruz P, Hanson E, McCole DF, Eckmann L. CXCR2-Dependent Mucosal Neutrophil Influx Protects against Colitis-Associated Diarrhea Caused by an Attaching/Effacing Lesion-Forming Bacterial Pathogen. J Immunol Baltim Md 1950. 2009 Sep 1;183(5):3332–43.

115. Basu R, O'Quinn DB, Silberger DJ, Schoeb TR, Fouser L, Ouyang W, et al. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. Immunity. 2012 Dec 14;37(6):1061–75.

116. Geddes K, Rubino SJ, Magalhaes JG, Streutker C, Le Bourhis L, Cho JH, et al. Identification of an innate T helper type 17 response to intestinal bacterial pathogens. Nat Med. 2011 Jun 12;17(7):837–44.

117. Simmons CP, Clare S, Ghaem-Maghami M, Uren TK, Rankin J, Huett A, et al. Central role for B lymphocytes and CD4+ T cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. Infect Immun. 2003 Sep;71(9):5077–86.

118. Sakamoto K, Kim YG, Hara H, Kamada N, Caballero-Flores G, Tolosano E, et al. IL-22 Controls Iron-Dependent Nutritional Immunity Against Systemic Bacterial Infections. Sci Immunol. 2017 Feb;2(8):eaai8371.

119. Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB. Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A. 2003 Jul 22;100(15):8951–6.

120. Sircili MP, Walters M, Trabulsi LR, Sperandio V. Modulation of Enteropathogenic *Escherichia coli* Virulence by Quorum Sensing. Infect Immun. 2004 Apr;72(4):2329–37.

121. Reading NC, Torres AG, Kendall MM, Hughes DT, Yamamoto K, Sperandio V. A novel two-component signaling system that activates transcription of an enterohemorrhagic *Escherichia coli* effector involved in remodeling of host actin. J Bacteriol. 2007 Mar;189(6):2468–76.

122. Njoroge JW, Nguyen Y, Curtis MM, Moreira CG, Sperandio V. Virulence meets metabolism: Cra and KdpE gene regulation in enterohemorrhagic *Escherichia coli*. mBio. 2012 Oct 16;3(5):e00280-00212.

123. Sperandio V, Torres AG, Kaper JB. Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli.* Mol Microbiol. 2002 Feb;43(3):809–21.

124. Rasko DA, Moreira CG, Li DR, Reading NC, Ritchie JM, Waldor MK, et al. Targeting QseC signaling and virulence for antibiotic development. Science. 2008 Aug 22;321(5892):1078–80.

125. Moreira CG, Weinshenker D, Sperandio V. QseC mediates *Salmonella* enterica serovar typhimurium virulence in vitro and in vivo. Infect Immun. 2010 Mar;78(3):914–26.

126. Njoroge J, Sperandio V. Enterohemorrhagic *Escherichia coli* virulence regulation by two bacterial adrenergic kinases, QseC and QseE. Infect Immun. 2012 Feb;80(2):688–703.

127. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, et al. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol. 2012 Dec 1;303(11):G1288-1295.

128. Strandwitz P. Neurotransmitter modulation by the gut microbiota. Brain Res. 2018 Aug 15;1693(Pt B):128–33.

129. Flierl MA, Rittirsch D, Huber-Lang M, Sarma JV, Ward PA. Catecholamines—Crafty Weapons in the Inflammatory Arsenal of Immune/Inflammatory Cells or Opening Pandora's Box? Mol Med. 2008 Mar;14(3):195–204.

130. Walters M, Sperandio V. Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic *Escherichia coli*. Infect Immun. 2006 Oct;74(10):5445–55.

131. Moreira CG, Russell R, Mishra AA, Narayanan S, Ritchie JM, Waldor MK, et al. Bacterial Adrenergic Sensors Regulate Virulence of Enteric Pathogens in the Gut. mBio. 2016 Jun 7;7(3):e00826-16.

132. Hoiseth SK, Stocker BA. Aromatic-dependent *Salmonella* Typhimurium are non-virulent and effective as live vaccines. Nature. 1981 May 21;291(5812):238–9.

133. Moreira CG, Sperandio V. Interplay between the QseC and QseE Bacterial Adrenergic Sensor Kinases in *Salmonella enterica* Serovar Typhimurium Pathogenesis. Infect Immun. 2012 Dec;80(12):4344–53.

134. Perez-Lopez A, Silva S, Lee MH, Dillon N, Brandt SL, Gerner RR, et al. CCL28 modulates neutrophil responses during infection with mucosal pathogens [Internet]. bioRxiv; 2022 [cited 2023 Nov 17]. p. 2021.03.19.436197. Available from: https://www.biorxiv.org/content/10.1101/2021.03.19.436197v2

135. Frey A, Di Canzio J, Zurakowski D. A statistically defined endpoint titer determination method for immunoassays. J Immunol Methods. 1998 Dec 1;221(1–2):35–41.

136. Cao DY, Giani JF, Veiras LC, Bernstein EA, Okwan-Duodu D, Ahmed F, et al. An ACE inhibitor reduces bactericidal activity of human neutrophils in vitro and impairs mouse neutrophil activity in vivo. Sci Transl Med. 2021 Jul 28;13(604):eabj2138.

137. Moschen AR, Gerner RR, Wang J, Klepsch V, Adolph TE, Reider SJ, et al. Lipocalin 2 Protects from Inflammation and Tumorigenesis Associated with Gut Microbiota Alterations. Cell Host Microbe. 2016 Apr 13;19(4):455–69.

138. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015;264(1):182–203.

139. Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O. Plague bacteria target immune cells during infection. Science. 2005 Sep 9;309(5741):1739–41.

140. Vareechon C, Zmina SE, Karmakar M, Pearlman E, Rietsch A. Pseudomonas aeruginosa Effector ExoS Inhibits ROS Production in Human Neutrophils. Cell Host Microbe. 2017 May 10;21(5):611-618.e5.

141. Kehl-Fie TE, Chitayat S, Hood MI, Damo S, Restrepo N, Garcia C, et al. Nutrient Metal Sequestration by Calprotectin Inhibits Bacterial Superoxide Defense, Enhancing Neutrophil Killing of *Staphylococcus aureus*. Cell Host Microbe. 2011 Aug 18;10(2):158–64.

142. Miyasaki KT, Bodeau AL, Murthy AR, Lehrer RI. In vitro antimicrobial activity of the human neutrophil cytosolic S-100 protein complex, calprotectin, against *Capnocytophaga sputigena*. J Dent Res. 1993 Feb;72(2):517–23.

143. Zygiel EM, Nolan EM. Transition Metal Sequestration by the Host-Defense Protein Calprotectin. Annu Rev Biochem. 2018 Jun 20;87:621–43.

144. Halverson TWR, Wilton M, Poon KKH, Petri B, Lewenza S. DNA Is an Antimicrobial Component of Neutrophil Extracellular Traps. PLoS Pathog. 2015 Jan 15;11(1):e1004593.

145. Corbett JA, Tilton RG, Chang K, Hasan KS, Ido Y, Wang JL, et al. Aminoguanidine,

a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. Diabetes. 1992 Apr;41(4):552–6.

146. Corbett JA, McDaniel ML. The Use of Aminoguanidine, a Selective iNOS Inhibitor, to Evaluate the Role of Nitric Oxide in the Development of Autoimmune Diabetes. Methods San Diego Calif. 1996 Aug;10(1):21–30.

147. Farhad AR, Razavi SM, Nejad PA. The use of aminoguanidine, a selective inducible nitric oxide synthase inhibitor, to evaluate the role of nitric oxide on periapical healing. Dent Res J. 2011;8(4):197–202.

148. Ruest MK, Dennis JJ. The Exploration of Complement-Resistance Mechanisms of Pathogenic Gram-Negative Bacteria to Support the Development of Novel Therapeutics. Pathogens. 2022 Aug 18;11(8):931.

149. West NP, Sansonetti P, Mounier J, Exley RM, Parsot C, Guadagnini S, et al. Optimization of virulence functions through glucosylation of Shigella LPS. Science. 2005 Feb 25;307(5713):1313–7.

150. Murray GL, Attridge SR, Morona R. Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of *Salmonella enterica* serovar Typhimurium with macrophages and complement. J Bacteriol. 2006 Apr;188(7):2735–9.

151. Maldonado RF, Sá-Correia I, Valvano MA. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. FEMS Microbiol Rev. 2016 Jul;40(4):480–93.

152. Berry MC, McGhee GC, Zhao Y, Sundin GW. Effect of a waaL mutation on lipopolysaccharide composition, oxidative stress survival, and virulence in *Erwinia amylovora*. FEMS Microbiol Lett. 2009 Feb;291(1):80–7.

153. Duerr CU, Zenk SF, Chassin C, Pott J, Gütle D, Hensel M, et al. O-antigen delays lipopolysaccharide recognition and impairs antibacterial host defense in murine intestinal epithelial cells. PLoS Pathog. 2009 Sep;5(9):e1000567.

154. Mevorach D. Opsonization of apoptotic cells. Implications for uptake and autoimmunity. Ann N Y Acad Sci. 2000;926:226–35.

155. Charles A Janeway J, Travers P, Walport M, Shlomchik MJ. The complement system and innate immunity. In: Immunobiology: The Immune System in Health and Disease 5th edition [Internet]. Garland Science; 2001 [cited 2023 Oct 15]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27100/

156. Lebeis SL, Bommarius B, Parkos CA, Sherman MA, Kalman D. TLR Signaling Mediated by MyD88 Is Required for a Protective Innate Immune Response by Neutrophils to *Citrobacter rodentium*. J Immunol. 2007 Jul 1;179(1):566–77.

157. Kamada N, Kim YG, Sham HP, Vallance BA, Puente JL, Martens EC, et al. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. Science. 2012 Jun 8;336(6086):1325–9.

158. Ozuna H, Uriarte SM, Demuth DR. The Hunger Games: *Aggregatibacter actinomycetemcomitans* Exploits Human Neutrophils As an Epinephrine Source for Survival. Front Immunol. 2021;12:707096.

159. De Araujo C, Balestrino D, Roth L, Charbonnel N, Forestier C. Quorum sensing affects biofilm formation through lipopolysaccharide synthesis in *Klebsiella pneumoniae*. Res Microbiol. 2010 Sep;161(7):595–603.

160. Mokrievich AN, Kondakova AN, Valade E, Platonov ME, Vakhrameeva GM, Shaikhutdinova RZ, et al. Biological properties and structure of the lipopolysaccharide of a vaccine strain of *Francisella tularensis* generated by inactivation of a quorum sensing system gene qseC. Biochem Mosc. 2010 Apr 1;75(4):443–51.

161. Wang Y, Huang Y, Chase RC, Li T, Ramai D, Li S, et al. Global Burden of Digestive Diseases: A Systematic Analysis of the Global Burden of Diseases Study, 1990 to 2019. Gastroenterology. 2023 Sep 1;165(3):773-783.e15.

162. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol. 2011

May;12(5):383–90.

163. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic Bacteria Direct Expression of an Intestinal Bactericidal Lectin. Science. 2006 Aug 25;313(5790):1126–30.

164. Shin JH, Seeley RJ. Reg3 Proteins as Gut Hormones? Endocrinology. 2019 May 9;160(6):1506–14.

165. Kinnebrew MA, Ubeda C, Zenewicz LA, Smith N, Flavell RA, Pamer EG. Bacterial flagellin stimulates TLR5-dependent defense against vancomycin-resistant Enterococcus infection. J Infect Dis. 2010 Feb 15;201(4):534–43.

166. Hasegawa M, Yada S, Zhen Liu M, Kamada N, Muñoz-Planillo R, Do N, et al. Interleukin-22 Regulates the Complement System to Promote Resistance against Pathobionts after Pathogen-Induced Intestinal Damage. Immunity. 2014 Oct 16;41(4):620–32.

167. Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature. 2004 Dec 16;432(7019):917–21.

168. Feinen B, Russell MW. Contrasting Roles of IL-22 and IL-17 in Murine Genital Tract Infection by Neisseria gonorrhoeae. Front Immunol. 2012;3:11.

169. Graham AC, Carr KD, Sieve AN, Indramohan M, Break TJ, Berg RE. IL-22 Production Is Regulated by IL-23 During Listeria monocytogenes Infection but Is Not Required for Bacterial Clearance or Tissue Protection. PLOS ONE. 2011 Feb 15;6(2):e17171.

170. Behnsen J, Jellbauer S, Wong CP, Edwards RA, George MD, Ouyang W, et al. The cytokine IL-22 promotes pathogen colonization by suppressing related commensal bacteria. Immunity. 2014 Feb 20;40(2):262–73.

171. Grizotte-Lake M, Zhong G, Duncan K, Kirkwood J, Iyer N, Smolenski I, et al.

Commensals Suppress Intestinal Epithelial Cell Retinoic Acid Synthesis to Regulate Interleukin-22 Activity and Prevent Microbial Dysbiosis. Immunity. 2018 Dec 18;49(6):1103-1115.e6.

172. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med. 2008 Mar;14(3):275–81.

173. Vallance BA, Deng W, Jacobson K, Finlay BB. Host Susceptibility to the Attaching and Effacing Bacterial Pathogen *Citrobacter rodentium*. Infect Immun. 2003 Jun;71(6):3443–53.

174. Tsai PY, Zhang B, He WQ, Zha JM, Odenwald MA, Singh G, et al. IL-22 Upregulates Epithelial Claudin-2 to Drive Diarrhea and Enteric Pathogen Clearance. Cell Host Microbe. 2017 Jun 14;21(6):671-681.e4.

175. Pham TAN, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. Cell Host Microbe. 2014 Oct 8;16(4):504–16.

176. Curtis MM, Hu Z, Klimko C, Narayanan S, Deberardinis R, Sperandio V. The gut commensal Bacteroides thetaiotaomicron exacerbates enteric infection through modification of the metabolic landscape. Cell Host Microbe. 2014 Dec 10;16(6):759–69.

177. Manitz MP, Horst B, Seeliger S, Strey A, Skryabin BV, Gunzer M, et al. Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. Mol Cell Biol. 2003 Feb;23(3):1034–43.

178. Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proc Natl Acad Sci U S A. 1995 Dec 5;92(25):11490–4.

179. Wang L, Fouts DE, Stärkel P, Hartmann P, Chen P, Llorente C, et al. Intestinal

REG3 Lectins Protect against Alcoholic Steatohepatitis by Reducing Mucosa-Associated Microbiota and Preventing Bacterial Translocation. Cell Host Microbe. 2016 Feb 10;19(2):227–39.

180. Wan Z, Fan Y, Liu X, Xue J, Han Z, Zhu C, et al. NLRP3 inflammasome promotes diabetes-induced endothelial inflammation and atherosclerosis. Diabetes Metab Syndr Obes Targets Ther. 2019 Sep 20;12:1931–42.

181. Overbergh L, Giulietti A, Valckx D, Decallonne B, Bouillon R, Mathieu C. The Use of Real-Time Reverse Transcriptase PCR for the Quantification of Cytokine Gene Expression. J Biomol Tech JBT. 2003 Mar;14(1):33–43.

182. Godinez I, Haneda T, Raffatellu M, George MD, Paixão TA, Rolán HG, et al. T cells help to amplify inflammatory responses induced by *Salmonella enterica* serotype Typhimurium in the intestinal mucosa. Infect Immun. 2008 May;76(5):2008–17.

183. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The Neutrophil Lipocalin NGAL Is a Bacteriostatic Agent that Interferes with Siderophore-Mediated Iron Acquisition. Mol Cell. 2002 Nov 1;10(5):1033–43.

184. Hood MI, Mortensen BL, Moore JL, Zhang Y, Kehl-Fie TE, Sugitani N, et al. Identification of an *Acinetobacter baumannii* Zinc Acquisition System that Facilitates Resistance to Calprotectin-mediated Zinc Sequestration. PLOS Pathog. 2012 Dec 6;8(12):e1003068.

185. Diaz-Ochoa VE, Lam D, Lee CS, Klaus S, Behnsen J, Liu JZ, et al. Salmonella Mitigates Oxidative Stress and Thrives in the Inflamed Gut by Evading Calprotectin-Mediated Manganese Sequestration. Cell Host Microbe. 2016 Jun 8;19(6):814–25.

186. Gaddy JA, Radin JN, Cullen TW, Chazin WJ, Skaar EP, Trent MS, et al. Helicobacter pylori Resists the Antimicrobial Activity of Calprotectin via Lipid A Modification and Associated Biofilm Formation. mBio. 2015 Dec 8;6(6):10.1128/mbio.01349-15.

187. Sanos SL, Vonarbourg C, Mortha A, Diefenbach A. Control of epithelial cell

function by interleukin-22-producing RORγt+ innate lymphoid cells. Immunology. 2011 Apr;132(4):453–65.

188. Brandl K, Plitas G, Schnabl B, DeMatteo RP, Pamer EG. MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp Med. 2007 Aug 6;204(8):1891–900.

189. Lo BC, Shin SB, Hernaez DC, Refaeli I, Yu HB, Goebeler V, et al. IL-22 Preserves Gut Epithelial Integrity and Promotes Disease Remission during Chronic *Salmonella* Infection. J Immunol. 2019 Feb 1;202(3):956–65.

190. Jones RG, Li X, Gray PD, Kuang J, Clayton F, Samowitz WS, et al. Conditional deletion of β1 integrins in the intestinal epithelium causes a loss of Hedgehog expression, intestinal hyperplasia, and early postnatal lethality. J Cell Biol. 2006 Nov 6;175(3):505–14.

191. Bouvard D, Brakebusch C, Gustafsson E, Aszódi A, Bengtsson T, Berna A, et al. Functional consequences of integrin gene mutations in mice. Circ Res. 2001 Aug 3;89(3):211–23.

192. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: Novel Integral Membrane Proteins Localizing at Tight Junctions with No Sequence Similarity to Occludin. J Cell Biol. 1998 Jun 29;141(7):1539–50.

193. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, et al. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol. 1993 Dec;123(6 Pt 2):1777–88.

194. Schneider MR, Dahlhoff M, Horst D, Hirschi B, Trülzsch K, Müller-Höcker J, et al. A Key Role for E-cadherin in Intestinal Homeostasis and Paneth Cell Maturation. PLoS ONE. 2010 Dec 14;5(12):e14325.

195. Gustafsson JK, Navabi N, Rodriguez-Piñeiro AM, Alomran AHA, Premaratne P, Fernandez HR, et al. Dynamic Changes in Mucus Thickness and Ion Secretion during *Citrobacter rodentium* Infection and Clearance. PLOS ONE. 2013 Dec 30;8(12):e84430.

196. Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, et al. Antibiotic Treatment Alters the Colonic Mucus Layer and Predisposes the Host to Exacerbated *Citrobacter rodentium*-Induced Colitis. Infect Immun. 2011 Apr;79(4):1536–45.

197. Parks OB, Pociask DA, Hodzic Z, Kolls JK, Good M. Interleukin-22 Signaling in the Regulation of Intestinal Health and Disease. Front Cell Dev Biol. 2015;3:85.

198. Zhu Q, Korenfeld D, Suarez-Fueyo A, Graham S, Jin L, Punit S, et al. Epithelial dysfunction is prevented by IL-22 treatment in a *Citrobacter rodentium*-induced colitis model that shares similarities with inflammatory bowel disease. Mucosal Immunol. 2022 Jun;15(6):1338–49.

199. Begg SL. The role of metal ions in the virulence and viability of bacterial pathogens. Biochem Soc Trans. 2019 Feb 28;47(1):77–87.

200. Sheldon JR, Laakso HA, Heinrichs DE. Iron Acquisition Strategies of Bacterial Pathogens. Microbiol Spectr. 2016 Mar 18;4(2):10.1128/microbiolspec.vmbf-0010–2015.

201. Gerner RR, Hossain S, Sargun A, Siada K, Norton GJ, Zheng T, et al. Siderophore Immunization Restricted Colonization of Adherent-Invasive *Escherichia coli* and Ameliorated Experimental Colitis. mBio. 2022 Sep 12;13(5):e02184-22.

202. Behnsen J, Zhi H, Aron AT, Subramanian V, Santus W, Lee MH, et al. Siderophore-mediated zinc acquisition enhances enterobacterial colonization of the inflamed gut. Nat Commun. 2021 Dec 1;12(1):7016.

203. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. Front Immunol. 2015 May 26;6:257.

204. Belzer C, Liu Q, Carroll MC, Bry L. The role of specific igG and complement in combating a primary mucosal infection of the gut epithelium. Eur J Microbiol Immunol. 2011 Dec;1(4):311–8.

205. Mukherjee S, Zheng H, Derebe MG, Callenberg KM, Partch CL, Rollins D, et al.

Antibacterial membrane attack by a pore-forming intestinal C-type lectin. Nature. 2014 Jan;505(7481):103–7.

206. Choi SM, McAleer JP, Zheng M, Pociask DA, Kaplan MH, Qin S, et al. Innate Stat3-mediated induction of the antimicrobial protein Reg3γ is required for host defense against MRSA pneumonia. J Exp Med. 2013 Mar 11;210(3):551–61.

207. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature. 2008 Oct 9;455(7214):804–7.

208. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med. 2008 Mar;14(3):282–9.

209. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest. 2008 Feb;118(2):534–44.

210. Bry L, Falk PG, Midtvedt T, Gordon JI. A Model of Host-Microbial Interactions in an Open Mammalian Ecosystem. Science. 1996 Sep 6;273(5280):1380–3.

211. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina T, et al. Rapid fucosylation of intestinal epithelium sustains host-commensal symbiosis in sickness. Nature. 2014 Oct 30;514(7524):638–41.