VALIDATING SALMONELLA TYPHIMURIUM VIRULENCE MODULATION BY ECTO-5'-NUCLEOTIDASE (CD73) IN INTESTINAL EPITHELIAL CELLS

by

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DEDICATION

I dedicate this thesis to my teachers who took the time to fill my heart and feed my head. Thank you for your encouragement, your patience, and your authenticity. I am forever grateful for so much love and kindness throughout the years.

And to Pod, for being with me every step of the way.

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ABSTRACT

Extracellular ATP is a pro-inflammatory molecule released during intestinal insult and must be converted to adenosine by ecto-5'-nucleotidase (CD73) for the resolution of intestinal inflammation [1]. Along with its anti-inflammatory role in the intestinal mucosa, CD73-generated adenosine contributes to host-microbe interactions at the mucosal surface by modulating pathogen replication and virulence, including that of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) [2], [3]. It has been shown, in the absence of intestinal epithelial cell-specific CD73, *S.* Typhimurium virulence is attenuated *in vivo* and *in vitro*, implicating the intestinal epithelium as an underappreciated source for the development of novel antimicrobial therapies. Since direct modulation of extracellular adenosine leads to pleiotropic effects, the aim of this research was to determine the mechanism(s) of *S.* Typhimurium virulence modulation by CD73 in intestinal epithelial cells to identify specific molecular targets that modulate pathogenesis [4], [5]

BACKGROUND

Part I: The Intestines

The human intestines include the segments of the digestive tract running from the pyloric sphincter of the stomach to the anus. The intestinal tract serves vital functions for food digestion, nutrient and water absorption, waste excretion, and plays significant roles in endocrine signaling and immunity [6]. The structure of the gut is often broken down into two general segments, the small and large intestines, as these segments differ in both anatomy and function. Furthermore, there is a large population of symbiotic bacteria housed in the gut lumen called the intestinal microbiome. While the small intestine houses some members of the gut microbiota, the bulk of this population resides in the large intestine [7].

Starting at the stomach, the small intestine includes three major divisions: the duodenum, jejunum, and ileum. The duodenum is the shortest segment of the small intestine but it is critically important for digestion, as this is where stomach contents, pancreatic secretions, bile, and intestinal brush border enzymes all converge to start intestinal digestion. Pancreatic secretions contain bicarbonate to finalize the neutralization of acidic stomach contents as well as digestive enzymes such as amylase, lipase, and trypsin to enzymatically break down carbohydrates, lipids, and proteins [8], [9]. Bile released from the gallbladder contains primary bile acids, which are synthesized in the liver, and secondary bile acids, which are derived from bacteria in the colon, as well as phospholipids and cholesterol, all of which help to emulsify fats for better absorption by intestinal epithelial cells [10]. Additionally, bile acids help to remove

waste products such as bilirubin and excess cholesterol and acts as a bactericidal agent and antimicrobial mediator, which keeps the abundance of intestinal microbial species low in the small intestine [11].

Brush border enzymes, such as lactase, sucrase, and aminopeptidase, are hydrolases anchored into intestinal epithelial cells and are present in the duodenum as well as the jejunum and proximal ileum [12]. The small intestine is where most chemical digestion and nutrient absorption occurs due to the presence of so many digestive enzymes and the large surface area of absorptive enterocytes in the small intestinal epithelium [13], [14]. The distal ileum is also where most bile acids are reabsorbed and transported back to the liver to reenter the enterohepatic circulation of bile acids [10]. Material that cannot be digested in or absorbed by the small intestine is passed on to the large intestine.

The cecum is the first structure of the large intestine and acts as the junction point for the ileum, appendix, and ascending colon. The appendix is often considered a vestigial structure since it is a dead-end pouch and is not involved in the typical flow of digested matter, but it has become widely accepted that the appendix may act as a reservoir of gut microbial species in times of dysbiosis [15]. From the ascending colon, the large intestine continues through to the transverse colon, descending colon, sigmoid colon, and, finally, the rectum, which connects to the anus. The colon serves as the primary site for water absorption and fecal matter compaction [16]. The large intestine is also important for the synthesis and absorption of several key nutrients and is a critical

site for immune maturation, homeostasis, and defense, in part due to interactions with commensal microbial species housed within the colon [17].

The histology of the intestines varies by segment, but generally includes four layers: the mucosa, submucosa, muscularis externa, and the adventitia or serosa. The mucosa is the innermost layer of the intestines that surrounds the gut lumen and is critically important for intestinal immune function and homeostasis as well as proper nutrient and water absorption [18]. The submucosa is made up almost entirely of collagen fibers and loosely connects the intestinal mucosa to the muscularis externa. The submucosa layer supports the mucosa and fosters communication by allowing the passage of lymphatics, an extensive vascular network, enteric nerves, and mucous secreting glands that are kept in place by the collagen fibers [19]. The muscularis externa contains both longitudinal and circular smooth muscle which coordinate peristalsis to move luminal contents through the intestinal tract. Finally, the muscularis externa is covered by one of two layers of loose connective tissue, either the rigid adventitia or lubricating serosa. The relatively immobile ascending colon, descending colon, duodenum, and rectum lie primarily in the retroperitoneal region and are covered by adventitia while other intestinal segments are contained in the peritoneum and are covered by serosa. These outermost layers are important for maintaining organ architecture and support larger blood vessels, lymphatic components, and nerve bundles that project to the lower layers of the intestinal wall [20].

Gut-associated lymphoid tissues (GALT) GALT are primarily situated within the mucosa and submucosa layers to aid in defense against pathogens and facilitate immune

tolerance to innocuous antigens. The presence of various immune-inductive GALT components, such as Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles vary throughout the length of the intestines and play a central role in local and systemic immune induction and tolerance [21]. Peyer's patches are well-organized groupings of lymphoid follicles that are located only along the length of the ileum and lie directly below the intestinal epithelium in the lamina propria. Mesenteric lymph nodes lie within the submucosa and collect draining lymph from the intestines for surveillance by antigen presenting cells (APCs) and stimulation of lymphocytes when necessary. Isolated lymphoid follicles are formed in response to antigenic stimulation by the intestinal microbiome and can form throughout the length of the intestines [22]. Lymphoid follicles are particularly well-organized and concentrated around the appendix, further lending support to the hypothesis that the appendix is important for intestinal immune function [15]. Additionally, there is an extensive vascular system that supports nutrient and blood cell flow throughout the intestines. It has recently been found that the endothelium of intestinal vasculature establishes a gut-vascular barrier (GVB), similar to the blood brain barrier, in order to prevent the dissemination of foreign antigens from the intestines to distal sites [23].

Continuous nerve ganglia of the enteric nervous system (ENS) are can also be found throughout the length of the intestines. There are two major enteric ganglia in the intestines that facilitate the signaling network of the ENS: the myenteric plexus, or Auerbach's plexus, which lies in between the circular and longitudinal muscles of the muscularis externa, and the submucosal plexus, or Meissner's plexus, which rests in the

submucosa. The enteric nervous system is partly controlled by the autonomic nervous system but can also operate autonomously from the brain and spinal cord to control reflex responses. The enteric nervous system has been coined the body's "second brain" due to its size, complexity, and functional importance, and it even has a dedicated endocrine system within the mucosa [24].

The mucosa can be further broken down into three distinct layers: the epithelium, lamina propria, and muscularis mucosae. The muscularis mucosae consists of several layers of smooth muscle and is the outermost portion of the mucosa. The muscularis mucosae, like the muscularis externa, uses longitudinal and circular muscle fibers to aid in peristaltic movements, but it mediates more intimate contact between the epithelium and luminal contents. This thin muscle layer helps to mobilize glandular secretions from intestinal crypts and agitate intestinal contents to improve contact between digestive enzymes and food particles as well as to enhance the uptake of absorbable nutrients by the intestinal epithelium [20].

The lamina propria is comprised of loosely-associated fibroblasts that act as a supportive mesh network for of a rather diverse population of leukocytes which take on many support, regulatory, and immune functions in the gut. The composition of the specific population of leukocytes in the mucosa varies by spatial location as well as disease state, but common cell types found in the lamina propria include lymphocytes, plasma cells, dendritic cells, macrophages, and a variety of granulocytes. Additionally, the lamina propria supports GALT and vascular tissues of the mucosa, which facilitates immune cell and nutrient exchange between the mucosa and underlying tissues [25]. The

lamina propria also helps to maintain the architecture of crypts throughout the gut as well as the integrity of projecting villi in the small intestine, although the formation and collapse of crypts and villi is thought to be dependent on signaling within the epithelium itself [26].

The Intestinal Epithelium

The intestinal epithelium is made up of a single layer of simple columnar epithelial cells that lie at the interface of the intestines and luminal contents. Arguably the most important function of the intestinal epithelium is to establish and regulate the barrier between underlying tissues and the milieu of nutrients, food antigens, water, waste products, and commensal and pathogenic microbes within the intestinal lumen [27]. Intestinal epithelial cells (IECs) are anchored together by several types of cellular junctions, which helps them to tightly control the influx and efflux of material across and throughout the intestinal barrier.

Cellular junctions in the intestinal epithelium include tight junctions, adherens junctions, desmosomes, and gap junctions. Tight junctions, or zonula occludens, are the cellular junctions that are most important for linking epithelial cells together and establishing a barrier between the intestinal lumen and underlying mucosa. Tight junctions are multiprotein complexes that consist primarily of claudin and occludin transmembrane proteins that lie in tightly-linked strands along the apical aspect of lateral cell membranes. Tight junctions regulate osmotic homeostasis in the gut by controlling the paracellular flux of ions, fluids, and other small molecules across the IEC barrier,

making the intestinal epithelium practically impenetrable to luminal contents when fully intact [28].

Adherens junctions, or zonula adherens, are another multiprotein complex made of cadherin and catenin proteins. Catenin proteins lie within IECs and anchor cadherin proteins to the cell while cadherins project from neighboring cells and link together in the extracellular space. Adherens junctions can be found slightly below tight junctions and their primary role is to anchor cells to one another [29], [30]. Desmosomes, or macula adherens, are another type of multiprotein complex that connect one cell to another via extracellular linkages between cadherin proteins. Multiple cadherins are grouped together in the plasma membrane and are anchored to a dense protein plaque on the cytosolic surface of cell membranes which link desmosomes to keratin fibers in the cytoskeleton [31].

While the other junctional proteins serve mainly to anchor cells to one another, the primary role of gap junctions is to facilitate the flow of small molecules and ions from one cell to another. Gap junctions are made up of two hemichannels which create a pore to connect the cytoplasm of one cell to another and in this way they do contribute to cell-to-cell attachment to a minor degree [32]. All of these cellular junctions are regulated by numerous signaling pathways that can fine-tune the flux of materials across and throughout of the intestinal epithelium [28].

The intestinal epithelium is a dynamic tissue consisting of a multitude of different IEC types and subtypes which all arise from a highly-active stem cell population located at the bottom of intestinal crypts. The intestinal epithelium is highly proliferative and is

renewed roughly every 3-5 days [33]. The typical process of epithelial turnover involves intestinal stem cells dividing in the base of intestinal crypts to give rise to IEC progenitor cells called transit amplifying (TA) cells. TA cells are located directly above the stem cell compartment and divide a finite number of times to give rise to the various epithelial cell types. As TA cells mature and then differentiate into different cell types, they continue to migrate up the crypt toward the lumen. IECs serve their function for several days and are finally shed from the top of the crypt in the colon or tip of the villus in the small intestine [33].

Epithelial cell shedding, proliferation, and differentiation must be tightly regulated to maintain the health and integrity of the intestinal mucosa, which is why it is perhaps not surprising that there have been at least two distinct intestinal stem cell populations identified. Crypt base columnar (CBC) intestinal stem cells express the R-spondin receptor *Lgr5* which is part of the *Wnt* signaling pathway. CBCs are considered to be the primary intestinal stem cell population based off lineage tracing experiments, their presence throughout the length of the digestive tract, and their relatively higher mitotic index compared to +4 intestinal stem cells [34], [35]. *Lgr5*- +4 cells are slower cycling cells found only in the crypts of the small intestine and are thought to serve as reserve intestinal stem cells for times of intestinal distress due to their ability to give rise to all IEC types and maintain intestinal homeostasis in the absence of *Lgr5* [34], [36].

Although their precise roles in development and disease are still being defined, CBCs and +4 cells are easy to distinguish histologically [37]. CBCs are observed in the base of both small intestinal and large intestinal crypts and are sandwiched between

Paneth cells in the small intestine. +4 cells reside just above Paneth cells in the +4 position relative to the base of the crypt [36]. Paneth cells are usually only found in the small intestine and help to constitute the intestinal stem cell niche there by providing growth factors and secreting antimicrobial peptides. Unlike most IECs which have a lifespan of several days, Paneth cells live for about 2 months and migrate downward to remain in intestinal crypts instead of moving toward the intestinal lumen [34], [38].

Absorptive enterocytes are the prototypical cell type of the intestinal epithelium, representing over 80% of all IECs, and providing most of the mechanical integrity for the epithelial barrier. Enterocytes are also responsible for the initial absorption and processing of nutrients from the intestinal lumen. While crypt and villus structures of the intestinal mucosa allow for a greatly increased surface area within the length of the intestine, enterocytes also have a brush border of microvilli projecting from their apical surface which further increases the absorptive and digestive surface area of each individual enterocyte [39].

Along with establishing a barrier between the intestinal lumen and the underlying mucosa by the way of junctional proteins, enterocytes have several other important features that facilitate innate and adaptive immune mechanisms in the gut. One way by which enterocytes contribute to immune tolerance and pathogen defense is through immune exclusion mechanisms, that is, preventing interactions between luminal antigens and host cells. Although goblet cells are the primary producers of mucus, enterocytes express several transmembrane mucins on their luminal surface which contribute to the protective mucus layer throughout the intestines [40]. Enterocytes can also transport

secretory IgA antibodies from plasma cells in the lamina propria to the mucosal surface [41]. Secretory IgA antibodies bind antigens in the intestinal lumen to limit the ability of these antigens to come into contact with the epithelium [42]. Enterocytes are also important for facilitating the detection of luminal antigens in the gut. Along with regulating the paracellular flux of fluids and small molecules, enterocytes regulate leukocyte extravasation across the epithelial cell barrier to allow for antigen sampling and the infiltration of immune effector cells into the intestinal lumen. Furthermore, enterocytes can act as antigen presenting cells themselves by endocytosing and processing luminal antigens for recognition by lymphocytes found in the lamina propria or between epithelial cells [43].

The population of lymphocytes found within the epithelium are called intraepithelial lymphocytes (IELs) and are situated in the basal aspect of the epithelial layer below tight junctional proteins [44]. Most IELs are T cells that are distinct from lamina propria lymphocytes both in their receptor expression and in immune function [45]. Like other cell types in the intestines, IELs are heterogeneous throughout the digestive tract, but one distinguishing feature of IELs is that most do not require priming to stimulate their effector functions [44]. IELs help to main barrier integrity through neutralizing or damaging harmful pathogens, regulating inflammatory responses, and contributing to the healing process of damaged epithelial cells [45].

M cells, or microfold cells, are localized just above Peyer's patches and other GALT components and make up the majority of the follicle associated epithelium. Although enterocytes are able to sample luminal antigens to a minor degree, the primary

role of M cells is to continually transcytose antigens from the lumen to the underlying lymphoid tissue [46]. Instead of microvilli, M cells exhibit smaller membranous folds without many of the pathogen defense mechanisms that are present on enterocytes. The specific structural and chemical features of M cells are thought to aid in their ability to endocytose microbial antigens which is an important element of immunosurveillance in the gut [46], [47]. However, the lack of defense mechanisms and the constant and rapid sampling of intestinal microbes leave M cells vulnerable to exploitation by some intestinal pathogens such as *Salmonella enterica* [47], [48].

Goblet cells, another IEC subset, are also important for host defense against pathogens and maintaining homeostasis in the epithelium. Goblet cells are known for their production and secretion of gel-forming mucins that establish protective mucus layer that prevents intimate contact between luminal contents and the epithelium [49]. In the small intestine, goblet cells generate a thin mucus layer that helps to create a barrier between the epithelium and microbes in the gut lumen while still allowing for the passage of nutrients and other small molecules [40]. Goblet cells are found in higher proportions in the colon compared to the small intestine and they produce two chemically distinct mucus layers which are necessary for minimizing contact between the more abundant bacterial load in the large intestine. The outer mucus layer in the colon has a looser mucin network that provides a habitat for commensal microbial species while the inner mucus layer that coats the epithelium is much thicker and harder to penetrate [49].

Enteroendocrine cells act as chemical sensors and regulators of the gut environment and they are the major IEC type responsible for relaying information about

the chemical contents of the intestinal lumen to local and distal cell types [50]. Enteroendocrine cells appear as discrete cells in the IEC monolayer and are dispersed throughout the intestinal tract to help coordinate changes in gut function in response to chemical stimuli from the lumen. These chemosensory cells detect the presence of various nutritious and harmful compounds via G protein-coupled receptors (GPCRs). GPCR ligation on enteroendocrine cells stimulates signaling cascades that leads to the release of signaling molecules from their basement membranes that can act in a paracrine or endocrine manner to regulate blood flow, motility, osmosis, and digestive secretions [51]. Numerous enteroendocrine cell subtypes have been identified and are primarily classified by the contents of their secretory granules.

The last two major IEC subsets, tuft cells and cup cells, are not often mentioned because their precise function in the epithelium is still not fully elucidated. Recent evidence now suggests that tuft cells may act as another chemosensory cell type in the epithelium, but in contrast to enteroendocrine cells, it is thought that tuft cells are important for stimulating adaptive immune responses to parasitic infections [52], [53]. The wine glass shape of epithelial cup cells in histological sections of the mucosa is their primary defining feature and since their function and significance remains largely unknown, cup cells will not be further discussed here [38].

The Intestinal Microbiome and Host Immunity

Interactions with gut microbial species of the small intestine and colon are critical for mounting appropriate immune defense and tolerance responses. Antigen presenting cells sample contents of the intestinal lumen, traffic these antigens to immune inductive

sites in the GALT, and present them to lymphocytes in order to direct immune responses to antigens taken from the gut mucosa [25]. Interactions with different antigenic stimuli from gut microbial species can induce cytokine secretion that influences lymphocyte activation and expansion of particular lymphocyte subsets. In this way, microbial species closely associated with the gut mucosa can direct immunomodulatory and immune stimulatory responses via the indirect selection of immune cell types [54]. After exiting the GALT, lymphocytes can traffic to immune effector sites of the gut mucosa if they express gut-homing receptors or they can enter into systemic vascular and lymphatic circulation to provide host immunity to microbes sampled from the gut [55].

Additionally, commensal bacterial species housed within the gut lumen provide another layer of host defense through a mechanism termed colonization resistance. Commensal microbes established within the intestinal tract form a relatively stable community of microorganisms that resist perturbations by foreign microbes [56]. New bacterial species that enter the gut must therefore compete with established communities for niche space in order to become residents of the intestinal tract [57]. Colonization resistance is particularly important in the context of intestinal pathogens since it is difficult to compete with established commensal species during intestinal homeostasis [58], [59]. Therefore, intestinal pathogens must take advantage of incidents of intestinal dysbiosis, such as those caused by antibiotic exposure or inflammatory responses to succeed in a host [60]–[62]. Also, commensal bacteria can influence antimicrobial peptide secretion and mucus production from the intestinal epithelium to further contribute to innate defenses against intestinal pathogens [63], [64].

Part II: *Salmonella enterica*

Salmonella enterica (S. enterica) are Gram-negative, rod-shaped, facultative intracellular pathogens and are one of the most common causes of enteric bacterial infections in humans and livestock worldwide. The severity of salmonellosis can range from mild gastroenteritis to life-threatening systemic infections, depending on both strain- and host-specific factors [65]. There are over 2,600 identified serotypes of *S. enterica* which are grouped into one of six different subspecies, although the majority of clinically and economically relevant serovars fall into subspecies *enterica.* Subspecies *enterica* includes serovars Typhi and Paratyphi, which cause enteric fever, as well as Enteritidis, Typhimurium, and many more non-typhoidal *Salmonella* (NTS) serotypes. NTS strains typically cause gut-limited infections in humans, but can cause systemic infections in vulnerable populations, although the factors that lead NTS strains to cause bacteremia remain to be fully defined [66]. Collectively, it is estimated that NTS serovars cause a healthcare cost burden of almost $3.7 billion annually in the United States and these costs are expected to increase with the rise of antibiotic-resistant isolates [35], [36].

S. enterica spp. have a diverse range of animal hosts and environmental reservoirs which can make mitigating *Salmonella* contamination difficult. Most *S. enterica* infections in humans are caused by ingesting contaminated food or water and a distinct set of physical and metabolic features allow for prevalent fecal-oral transmission and survival through diverse environments [67]. Most notably, *S. enterica spp.* utilize two distinct type III secretion systems (T3SS) encoded in integrative gene elements termed Salmonella Pathogenicity Islands (SPIs) that are differentially expressed throughout the

course of infection. These T3SS apparatuses and their associated effector proteins are essential to *S. enterica* virulence, as they modulate inflammatory responses, coordinate cell invasion, and establish an intracellular niche that allows for propagation within host cells [68]–[70]. Secreted virulence effectors may be encoded within the same SPI as the T3SS that is responsible for translocating them across the bacterial cell wall, but it is also common to observe elements of multiple SPIs being expressed in tandem to coordinate *S. enterica* infection [71], [72].

Other noteworthy factors that contribute to the virulent and avirulent colonization of *S. enterica* include adhesin molecules, flagella, and a highly flexible metabolism. Adhesins expressed on the cell surface help *S. enterica* to persist on both biological and non-biological surfaces which is important both for success within vertebrate hosts as well as host to host transmission events. Adhesins also help to fine-tune the virulence potential of different *S. enterica* serovars. Some adhesins help facilitate intimate contact with the intestinal epithelium to prepare for intracellular invasion, while others encourage long-term persistence within a host leading to a chronic *Salmonella* carrier state in some individuals [73]–[75]. Flagella are also important in various stages of the *S. enterica* infectious cycle since they are critical appendages for dissemination within and between hosts and can also stimulate host immune responses [76]. Finally, metabolic adaptability is critical to the success of *S. enterica* between and within host organisms. *S. enterica* has been long-recognized for its ability to persist in water, produce, and feces, which all present different environmental challenges [77]. *S. enterica* must also live through the many caustic environments of the digestive tract, outcompete members of the intestinal

microbiome, and evade antimicrobial responses in order to complete its infectious cycle [60], [78], [79]. The development of effective antimicrobial agents against *S. enterica* has been challenging due to this metabolic versatility and is largely why *S. enterica spp.* remains an area of intense investigation [80].

Salmonella enterica subspecies *enterica* serovar Typhimurium

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) is the most well-studied *S. enterica* serovar. *S.* Typhimurium exhibits a broad host range, including humans, livestock, and mice, among others [65]. *S.* Typhimurium is the second most commonly-isolated *S. enterica* serovar and is a well-established model for interrogating host interactions with invasive inflammatory pathogens. In humans, *S.* Typhimurium typically causes a gut-limited infection and patients recover without treatment [66]. However, for those patients who develop severe gastroenteritis or typhoid-like symptoms, treatment strategies are becoming increasingly limited [80], [81]. In fact, *S.* Typhimurium has been recently classified as a serious public health threat due to high disease prevalence, rising incidence of antibiotic resistance, and its ability to confer this resistance to related *Enterobacteriacea* [78], [82], [83].

To establish infection in the intestinal epithelium, *S. enterica* must first overcome colonization resistance mechanisms employed by members of the commensal gut microbiota. The ability to stimulate an inflammatory response and thrive in inflammatory conditions are key to the success of *S.* Typhimurium in overcoming colonization resistance and breaching the intestinal epithelial barrier [83]. Bacterial LPS, flagella, and the SPI-1 and -2 T3SSs of *S.* Typhimurium are all implicated in triggering intestinal

inflammation that is sufficient to alter the host microbiota and allow *S.* Typhimurium to overcome colonization resistance [83], [84]. Upon stimulating or encountering an inflammatory response, *S.* Typhimurium has several fitness advantages over many commensal bacterial species. When innate immune cells transmigrate into the intestinal lumen and release reactive oxygen species, they inadvertently generate tetrathionate from thiosulfate, which enables *S.* Typhimurium to utilize ethanolamine as a carbon source to out-compete the resident microbiome [85], [86]. Ethanolamine is derived from the phospholipid membranes of extruded enterocytes and is abundant in the intestinal lumen, but in most circumstances, ethanolamine is not bioavailable to intestinal bacteria [86]. Additionally, stimulated enterocytes as well as infiltrating leukocytes and platelets release high amounts of ATP in the intestinal lumen which inhibits the growth of Gram-positive species of the intestinal microbiota but not *S.* Typhimurium [87]–[89]. Extracellular ATP released into the intestinal lumen can also exacerbate inflammatory responses from the mucosa leading to increased intestinal dysbiosis and tissue damage [90].

Once in close proximity to the epithelium, *S.* Typhimurium may utilize both passive and active modes of invasion to breach the intestinal epithelial barrier. First, *S.* Typhimurium can be passively trafficked across the epithelium by dendritic cells and M cells sampling the intestinal lumen [48], [57], [78]. In this way, *S.* Typhimurium expends little energy in gaining entry to the cells of the underlying vascular and lymphatics systems, but this mode of infection is often a less successful strategy for *S.* Typhimurium propagation in immune-competent individuals [91], [92]. A more successful strategy for

S. Typhimurium spread is to invade intestinal epithelial cells and take advantage of the nutrient-rich and relatively innocuous intracellular niche of the intestinal epithelium [93]–[95].

The SPI-1 T3SS apparatus as well as virulence-associated effector proteins are generally associated with invasion and priming the intracellular environment for *Salmonella* colonization. SPI-1 is most highly expressed upon contact with the intestinal epithelium *in vivo* and is usually accompanied by expression of flagella for efficient IEC invasion [96]. Upon binding to a host cell, wild-type *S.* Typhimurium injects SPI-1 effectors that control actin rearrangements in the cytoplasm in order to induce host membrane ruffling and uptake of *Salmonella* into a *Salmonella*-containing vacuole (SCV) [97]. While there is SPI-1-independent invasion, it is not well-characterized or understood [94], [98].

Once *S.* Typhimurium has entered IECs and established itself in an SCV, Salmonella infection can progress in two ways: 1) *Salmonella* may remain in an SCV where T3SS2-secreted effectors protect *Salmonella* from typical endocytic processes and direct trafficking through the host cell or 2) *Salmonella* can escape the SCV to hyper-replicate in the cytosol where SPI-1 and flagella are again upregulated and prime the *Salmonella* for further host cell invasion [95], [99], [100]. Replication within an SCV has been extensively studied due to the importance of SCV-dependent replication in macrophages and other phagocytic cells, but it is becoming clear that cytosolic replication represents an important strategy for *S.* Typhimurium virulence *in vivo* and *in vitro* [93]–[95], [97]. Intracellular replication within in an SCV ultimately produces fewer

replicating bacteria than cytosolic replication, but these bacteria are better at evading host immune responses [95], [101]

Host Responses to *S.* Typhimurium in the Intestinal Mucosa

There are both passive and active mechanisms of host immunity that act against *S.* Typhimurium in the intestinal mucosa. Passive responses include immune exclusion provided by secretory IgA, formation of thick mucus layers that are difficult to penetrate, secretion of antimicrobial peptides, which damage the bacterial membrane, and the establishment of an IEC barrier that prevents passive dissemination across the epithelium. Active mechanisms of immunity against *S.* Typhimurium are largely dependent upon pattern recognition receptor (PRR) ligation to stimulate inflammatory responses [78].

PRRs can recognize *S.* Typhimurium pathogen-associated molecular patterns (PAMPS) in the extracellular space, in endosomes, phagosomes, and in the cell cytosol. Toll-like receptors (TLR) 1, 2, and 6 are expressed on the external cell surface and work together to recognize *Salmonella*-derived lipoproteins [102]. The TLR1/2 heterodimer can also recognize amyloid curli fibers produced by *S.* Typhimurium during biofilm formation [103]. TLR5 is also expressed on the surface of enterocytes and binds *S.* Typhimurium flagellin [104]. TLR4 can recognize its ligand, LPS, both on the cell surface and within endosomes/phagosomes [102]. TLR9 can recognize CpG islands in the *S.* Typhimurium genome and is also present in the endosome/phagosome compartment [78]. All of these TLRs are present in a heterogenous manner along the crypt-villus and longitudinal axes of the intestinal epithelium, as well as in different populations of hematopoietic cells in the mucosa [105].

With the exception of endosomal TLR4, *Salmonella* PAMP ligation to TLRs leads to the recruitment of cytosolic adaptor proteins through a MYD88-dependent mechanism which initiates a signaling cascade that ultimately leads to NFkB nuclear translocation [102]. NFkB transcriptional activation results in the production of large amounts of IL-8 from the intestinal epithelium in response to *S.* Typhimurium detection [106]–[108]. IL-8 is also known as neutrophil chemotactic factor and is the basis of the neutrophil-dominant immune response to *S.* Typhimurium in the intestinal mucosa [59], [106]. In addition to stimulating the MYD88 pathway at the cell surface, TLR4 that is taken into phagosomes and endosomes utilizes the cytosolic adaptor molecule TRIF to stimulate a signaling cascade that results in a type-1 interferon (IFN) response [109]. TLR ligation also primes the cell for inflammasome activation, which is another anti-*Salmonella* immune mechanism that is activated when *S.* Typhimurium PAMPs are detected in the cytosol.

Inflammasomes are multiprotein signaling complexes that are formed in response to cytosolic NOD-like receptor (NLR) ligation. NLRs are cytosolic PRRs which can respond to both microbial PAMPs as well as endogenous damage-associated molecular patterns (DAMPs), such as extracellular ATP [110]. Upon detecting PAMP and DAMP activating signals, NLRs recruit caspases and other adaptor proteins to stimulate a signaling cascade that results in the activation of caspase-1. Active caspase-1 can then cleave the pro-forms of IL-1β and IL-18 into their active cytokines, and can also stimulate pyroptosis pathways is the cell [78], [111]. *Salmonella* uptake into an SCV

prevents caspase-1 activation and stimulation of pyroptosis since microbial PAMPs are hidden from sentinel NLRs [112].

Pyroptosis is a highly inflammatory form of programmed cell death that is characterized by plasma membrane rupture and release of intracellular contents. Cell lysis by pyroptosis provides a wealth of inflammatory signals, including proinflammatory cytokines, extracellular ATP, and other DAMPs, to nearby cells in order to propagate the inflammatory response against pathogens [112], [113]. *S.* Typhimurium can activate pyroptosis using the NLRC4 inflammasome as well as a non-canonical caspase-4 (-11) inflammasome in the intestinal epithelium [78], [111], [114], [115]. The non-canonical caspase-4 (-11) inflammasome may or may not involve NLRP3 in IECs and is thought to be stimulated by cytosolic LPS, although a specific activating signal and full mechanism has yet to be defined [116]–[118].

The NLRC4 inflammasome is activated by components of T3SSs and flagella, and is important for limiting *Salmonella* dissemination in both epithelial cells and immune cells in the lamina propria [115]. In the epithelium, specifically, NLRC4 inflammasome activation leads to infected IECs being extruded from the cell monolayer [119]. IEC extrusion may serve as a host defense mechanism to limit pathogen spread initially, *S.* Typhimurium can sometimes take advantage of this mechanism. *Salmonella* that escape their SCV to replicate in the cytosol are upregulated for SPI-1 and flagella, which primes them for invasion [93]. Therefore, when IECs containing cytosolic *Salmonella* undergo pyroptosis and are extruded from the monolayer, the intestines are

re-seeded with an abundance of invasion-primed *Salmonella* which thrive in the associated inflammatory environment of the intestinal lumen [93].

Since *Salmonella* is relatively successful at subverting the innate immune responses of the epithelium and recruited neutrophils, other innate and adaptive immune responses must be relied upon to prevent *Salmonella* dissemination from the intestines to distal sites [93], [120]. For example, LPS from *S.* Typhimurium is able to activate the complement cascade which can initiate the assembly of the membrane attack complex and lead to bacterial cell lysis [121], [122]. Opsonization of *Salmonella* by complement and anti-*Salmonella* antibodies also enhances phagocytosis and microbial killing by macrophages in the mucosa [122], [123]. IFNγ release by Th1 cells in the lamina propria further increases *S.* Typhimurium killing in macrophages [122]. Additionally, it has been shown that when *S.* Typhimurium is phagocytosed by dendritic cells and trafficked to the GALT, this acts as a confinement mechanism that simultaneously prevents systemic dissemination and allows for adaptive immune responses to take over and protect the host against further *S.* Typhimurium infection [91].

Laboratory models to study *S.* Typhimurium in Intestinal Epithelial Cells

The Gentamicin Protection Assay: The gentamicin protection assay can be used to assess the ability for *S.* Typhimurium to invade and replicate inside intestinal epithelial cells *in vitro* [124]. *S.* Typhimurium is grown to late-log phase in order to induce the expression of SPI-1 and flagella to maximize invasion of IECs [125]. Bacteria are then applied to the IEC monolayer at the desired multiplicity of infection (MOI), given several minutes to attach to IECs, and then non-adherent *Salmonella* are washed from the cell

surface. After further incubation in antibiotic-free base cell media to allow for bacterial internalization, media containing a high concentration of gentamicin, typically 50-100μg/mL, is applied for 30 minutes to an hour in order to kill off extracellular bacteria; *Salmonella* that have successfully invaded IECs are protected from gentamicin-induced killing in the intracellular space [126].

After this point, cells are maintained in media containing a lower concentration of gentamicin, typically 5-10μg, to prevent extracellular replication of escaped bacteria without inhibiting the growth of intracellular *Salmonella.* These infected cell monolayers can then be used in a multitude of ways, but methods most commonly include fixation for microscopy experiments, processing for gene expression analyses, or enumeration of intracellular bacteria by selectively solubilizing IEC plasma membranes. *Salmonella* invasion is typically measured at 1-1.5 hours post-infection (h.p.i.) whereas intracellular replication is measured at any time after 4.5 h.p.i. [124].

The Streptomycin Pretreatment Model of Salmonella Colitis: The streptomycin pretreatment model of Salmonella colitis is widely used to establish S. Typhimurium infection that recapitulates many of the hallmarks of human intestinal pathology in mice [59]. In the absence of streptomycin pretreatment, immune competent, wild-type mice develop pathologies more characteristic of human typhoid fever without the associated intestinal inflammation [58]. However, pretreating mice with streptomycin 24 hours before S. Typhimurium inoculation causes intestinal dysbioses that allow for the assessment of host and pathogen factors that contribute to non-typhoidal salmonellosis in mice [59].

In the streptomycin pretreatment model, disease progression is commonly assessed by tracking body weight throughout the course of infection and assessing murine tissues for host responses and *Salmonella* colonization post-mortem. Common readouts include histological scoring of intestinal samples, assessment of immune cell infiltration to the intestinal mucosa, measurement of colon length, quantification of *Salmonella* or mouse gene expression, and enumeration of the bacterial load in stool, mesenteric lymph nodes, liver, spleen, and intestinal tissue samples. The most severe pathology is observed in the cecum and colon in the streptomycin-pretreatment model of *Salmonella* colitis [127].

In order to study host-pathogen responses in the intestinal epithelium specifically, IEC-specific conditional knockout mouse models can be used in the streptomycin-pretreatment model. IEC-specific knockout mice can be generated by breeding mice harboring a Cre recombinase under the control of the Villin-1 promoter with mice containing loxP sites flanking a gene of interest [128]. Villin is an actin-binding protein that is expressed in all intestinal epithelial cells, and therefore, Cre-Lox recombination primarily occurs in the intestinal epithelium of floxed, $Villin^{Cre}$ mice [129].

Part III: Ecto-5'-nucleotidase (CD73) Limits Pro-Inflammatory Responses to Pathogens in the Intestinal Mucosa

CD73 is a dimeric, glycosylphosphatidylinositol (GPI)-anchored plasma membrane surface enzyme that is critical in the conversion of proinflammatory mediators toward those that direct anti-inflammatory responses in the extracellular space [1], [2], [5]. CD73 is highly expressed on the apical surface of IECs and most immune cell types

in the lamina propria and is the rate-limiting enzyme in the metabolism of extracellular ATP to adenosine [130]–[132]. When ATP is released into the extracellular space, it is metabolized to AMP by ecto-apyrase (CD39), and then extracellular AMP is quickly hydrolyzed to adenosine by CD73 [4], [133]. Although extracellular ATP serves a DAMP and propagates inflammatory actions, adenosine primarily exerts tissue protective effects in the intestinal mucosa by stimulating immunosuppressive pathways and increasing nutrient flow to injured tissues [134], [135]. Therefore, the concerted actions of CD39 and CD73 act to quench inflammatory responses for the resolution of inflammation in the intestinal mucosa [1], [131].

ATP is released into the intestinal lumen by pathogenic bacteria themselves, hematopoietic cells upon pathogen detection, and infected IECs [84], [87], [136]–[138]. Some enteric pathogens, such as *S.* Typhimurium, have evolved mechanisms to exploit ATP-associated inflammatory responses to intensify virulence at various times during their infectious cycle [83], [85], [86]. Additionally, excessive inflammation can result in tissue damage and increased pathology in host tissues [1], [90], [139]. In fact, it has been shown that CD73-generated adenosine, specifically, is a requirement for resolving colonic inflammation in mice [1], [5].

However, abolishment of ATP-stimulated inflammatory responses can allow pathogens to disseminate undetected throughout host tissues and prolong infection [131], [140]–[142]. This was exemplified in two studies by Alam et al. who demonstrated the importance of a strong ATP-associated inflammatory response for pathogen clearance [2]. In one study, these authors measured a decrease in CD39 and CD73 transcript

expression in response to *S.* Typhimurium infection, which was associated with an increase in pro-inflammatory cytokine production. Although one might have expected an increased inflammatory response to correspond with heightened *Salmonella* virulence, they went on to show that mice lacking CD73 ($CD73^{-/-}$) were better at limiting pathogen spread from the intestines. Therefore, although there was less intestinal tissue damage in response to *S.* Typhimurium in wild-type mice, $CD73^{-/-}$ mice were better at preventing systemic dissemination of the bacteria [2]. In accordance with these findings, Alam and colleagues also conducted similar experiments with *Helicobacter* to demonstrate a similar detrimental effect of CD73-generated adenosine in response to bacterial infection [143]. Thus, the activity of CD73 in generating adenosine from ATP plays a critical role in limiting tissue damage in the gut mucosa, but can also allow pathogens to evade host immune mechanisms [1], [2], [131].

CD73 Regulates *S.* Typhimurium Virulence in Intestinal Epithelial Cells

Alam and colleagues used a mouse model with a full-body knockout of CD73 to demonstrate that CD73-generated adenosine decreases host resistance to *S.* Typhimurium *in vivo* [2]. However, since CD73 is globally expressed in mucosal tissues, these authors were unable to determine cell type-specific interactions in these mice [2], [131]. Because extracellular ATP and adenosine can modulate a multitude of different immune pathways in epithelial cells, endothelial cells, and hematopoietic cells throughout the body, only a tissue-specific understanding of which inflammatory mediators are damaging or supportive to *Salmonella enterica* virulence will lead to better approaches for treating and preventing salmonellosis [5], [131]. To address this knowledge gap, work in our lab by

Kao and others illuminated an IEC-specific role for CD7-generated adenosine in modulating *S.* Typhimurium virulence *in vivo* and *in vitro* [3].

Since ATP inhibits growth of many aerobic and anaerobic commensal bacterial species while only minimally inhibiting the growth of *S.* Typhimurium, they first wanted understand if adenosine or other purine metabolites exert any inhibitory or stimulatory effects on *Salmonella* replication and colonization [88], [89]. *S.* Typhimurium growth curve experiments demonstrated adenosine, but not upstream or downstream metabolites, inhibits the growth of *S.* Typhimurium. Additionally, they were able to support their findings *in vivo* by using a novel mouse model in which CD73 is only absent in the intestinal epithelium ($CD73^{f/f}$ $Villin^{Cre}$) in *Salmonella* challenge experiments. Using this mouse model, it was shown that *S.* Typhimurium bacterial load was increased in luminal contents of mice lacking CD73 in the intestinal epithelium (Cre(+)) but not in littermate controls (Cre(-)). These results taken together demonstrate an inhibitory effect of CD73-generated adenosine on *Salmonella* intestinal colonization [3].

On the other hand, although CD73-generated adenosine was inhibitory to *S.* Typhimurium replication in the lumen, Kao demonstrated that *S.* Typhimurium was better at colonizing host tissues in the presence of CD73 in IECs, which was in accordance with the study by Alam and colleagues [2]. *In vitro* findings confirmed that *S.* Typhimurium can invade, but not robustly replicate, human IECs lacking CD73. Interestingly, fluorescence microscopy experiments revealed that upon invasion, *S.* Typhimurium remains localized to the apical surface in IECs lacking CD73 instead of trafficking to the basolateral aspect [3]. Since the primary function of CD73 is to generate

extracellular adenosine, these studies suggest the inability of *Salmonella* to replicate in IECs lacking CD73 is due to differences in extracellular adenosine generation or signaling [144]. However, the mechanism(s) by which CD73 modulates intracellular replication of *S.* Typhimurium remain to be elucidated.

Hypotheses

Previous work in our lab has shown, that in the absence of CD73, S. Typhimurium is able to invade cultured IECs but becomes trapped at the apical surface, suggesting these cells are unable to support bacterial trafficking and replication. I hypothesize that in the absence of intestinal epithelial CD73, extracellular adenosine concentrations are decreased and extracellular ATP concentrations are increased, which leads to compensatory changes in gene regulation and metabolite generation in IECs. To test this hypothesis, I will measure intracellular and extracellular adenine nucleotide metabolite concentrations in CD73 knockdown and control human IECs before and throughout infection with S. Typhimurium. I will also measure changes in expression for genes associated with adenosine generation and transport as well as quantify changes in activity for enzymes involved in adenosine signaling.

I also hypothesize that alterations in cellular metabolism in the absence of CD73 limit the ability for S. Typhimurium virulence-associated genes to target host proteins and cause inflammatory disease in the epithelium. To test this hypothesis, I will examine the expression and spatial location of S. Typhimurium effector proteins and host proteins involved in propagating S. Typhimurium infection in CD73 knockdown and control human IECs before and during infection. I will also utilize S. Typhimurium mutants,

inhibitory drugs, and neutralizing antibodies to confirm candidate virulence attenuation mechanisms. Finally, I will use $CD73^{f/f}$ $Villin^{Cre}$ mice to compare S. Typhimurium infection dynamics and to validate *in vitro* findings. These tools will allow me to outline mechanisms by which CD73 absence in the epithelium decreases S. Typhimurium virulence.

VALIDATING THE ROLE OF CD73 IN SALMONELLA INFECTION

Introduction

The long-term goal for this project is to elucidate the mechanisms by which adenosine generated by the intestinal epithelium modulates inflammatory diseases. Intestinal epithelial cells (IECs) create a critical boundary between antigens in the intestinal lumen and the underlying submucosa [27]. Enteric pathogens such as *Salmonella enterica* initiate infection by overcoming colonization resistance by the gut microbiome and breaching the IEC barrier to cause inflammatory disease [60]. Thus, IECs represent a key point of host defense against pathogens. ATP is released into the intestinal lumen by enteric pathogens, IECs, and infiltrating immune cells and acts as a pro-inflammatory signal during infection [87], [90], [136], [137]. Once near the apical surface of intestinal epithelial cells, extracellular ATP is quickly converted to AMP by ecto-apyrase and other ATP and ADP hydrolases [131]. AMP must then be converted to the anti-inflammatory molecule adenosine by ecto-5'-nucleotidase (CD73) for the resolution of intestinal inflammation [1]. However, there is a critical knowledge gap in understanding the impact of CD73-generated adenosine on mechanisms of host-microbe interactions in the intestinal epithelium.

Work from our lab has demonstrated *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) pathogenesis is altered in the absence of IEC-specific CD73 *in vitro* and *in vivo* [3]. Although a previous report established a role for global CD73 expression in *Salmonella* virulence, this study was the first to look into tissue-specific effects of CD73 on *S.* Typhimurium pathogenesis [2], [3]. To understand the role of CD73 in IECs

in vitro, our lab generated T84 human colonic intestinal epithelial cells with a lentiviral-transduced short hairpin knockdown of CD73 (shCD73) and matched short hairpin non-target control cells (shNTC). T84 cells were chosen for our IEC model system since they naturally express a high level of CD73 on their apical surface [130]. Using the shCD73 and shNTC T84 cells in a gentamicin protection assay to assess *S.* Typhimurium invasion and intracellular replication, it was shown that *S.* Typhimurium was able to invade, but not robustly replicate, in shCD73 T84 monolayers compared to shNTC cells. Fluorescence microscopy experiments revealed that *S.* Typhimurium remains localized to the apical aspect of shCD73 T84s after invasion, but the mechanisms and details of *Salmonella* entry in the absence of CD73 in IECs remain to be defined [3].

Our lab also possesses a novel mouse line harboring an IEC-specific knockout of CD73 ($CD73^{f/f}$ $Villin^{Cre}$) to better understand the influence of CD73 in the intestinal epithelium *in vivo*. All mice in the $CD73^{f/f}$ $Villin^{Cre}$ colony harbor loxP sites within the CD73 gene, but only some mice inherit the Cre recombinase under the control of the Villin-1 promoter, allowing for fully-matched Cre (+) and Cre (-) littermate controls [3], [4]. Since the Villin-1 gene is expressed in all cells of the intestinal epithelium, $Villin^{Cre}$ allows for the conditional knockout of CD73 from the intestinal epithelium in Cre (+) mice [128]. Using $CD73^{f/f}$ $Villin^{Cre}$ mice in the streptomycin-pretreatment model of *Salmonella* colitis, it was found that the *S.* Typhimurium bacterial load is increased in the feces of Cre (+) mice. Finding more *S.* Typhimurium in luminal contents was unsurprising since our lab also demonstrated an inhibitory effect of adenosine on *S.* Typhimurium growth *in vitro.* However, even though Cre (+) mice housed more

Salmonella in their luminal contents, they exhibited decreased pathology from *Salmonella* colitis as measured by weight loss, colon shortening, and histochemical staining of colon sections compared to Cre (-) control mice [3]. These results taken together suggest CD73-generated adenosine from IECs serves as an important virulence mediator for *S.* Typhimurium *in vitro* as well as *in vivo.*

These studies by Kao et al. established a role for IEC-specific CD73 in *S.* Typhimurium virulence, but the specific factors leading to virulence attenuation by IECs lacking CD73 remained elusive. For example, it was unknown whether *S.* Typhimurium was able to modulate and recruit all the associated host proteins to form an intact SCV when invading IECs lacking CD73. It was also unclear whether the infection phenotype that was observed was due to acute alterations in CD73-generated adenosine levels or if the *S.* Typhimurium virulence attenuation in IECs lacking CD73 was an effect of long-term compensatory changes made in epithelial cells lacking CD73. Furthermore, *in vitro* experiments were conducted in a single cell culture model that was not extensively used to study *S.* Typhimurium and the results of these experiments had not yet been repeated in another facility by other researchers. Therefore, the goal of the research outlined in this thesis was to validate the *in vitro* and *in vivo* results reported by Kao et al. and to extend these findings by elucidating the mechanism(s) by which CD73 modulates *S.* Typhimurium virulence in the intestinal epithelium.

Materials and Methods

Salmonella Typhimurium

Growth and Maintenance: Wild-type *Salmonella* enterica subsp. *enterica* serovar Typhimurium SL1344 (*S.* Typhimurium SL1344) and *S.* Typhimurium with an ampicillin-resistant mCherry plasmid (*S.* Typhimurium mCherry) were obtained as a gift from Dr. C. Detweiler, University of Colorado [3]**.** The wild-type SL1344 strain was also obtained as a gift from Dr. L. Knodler, Washington State University (*S.* Typhimurium SL1344W). In all *S.* Typhimurium culture conditions, bacteria were grown in the presence of 100µg/mL streptomycin (Thermo Fisher Scientific). SL1344 mCherry cultures were also supplemented with 50µg/mL ampicillin (RPI, Mt. Prospect, IL) to maintain the mCherry plasmid. Upon receipt of stab cultures, all strains were made into fresh freezer stocks by mixing 500µL of an overnight culture (see below) with 500µL 50% glycerol (Fisher Scientific, Fair Lawn, NJ) in dH_2O in a 2mL cryovial before storing at -80°C.

For preparation of working cultures, bacteria were streaked from freezer stocks onto LB-Miller agar plates (Fisher Scientific, Belgium) and grown overnight at 37°C. Fresh streak plates were prepared each week and kept at 4°C. Overnight *S.* Typhimurium liquid cultures were prepared by inoculating a single colony into 2mL LB-Miller broth (Fisher Scientific, Fair Lawn, NJ) with antibiotics in a loosely capped 14mL polypropylene round-bottom test tube and grown in a shaking incubator (225 r.p.m.) at 37°C for 16-18 hours. For all experimental cultures, 300µL of overnight culture was inoculated into 10mL LB-Miller broth supplemented with antibiotics in a 125mL glass

Erlenmeyer flask and grown at 37°C in the shaking incubator until mid-log phase (2.5 hours) or late-log phase (3.5 hours). Control broth cultures, containing LB-Miller broth and antibiotics alone, were always grown alongside overnight and experimental cultures to ensure sterility of reagents.

Standard Curve/Growth Curve Generation and Inocula Preparation: For preparation of *S.* Typhimurium standard curves and growth curves, triplicate 1mL samples were taken every 20-30 minutes for colony enumeration along with duplicate 1mL samples taken for OD600 measurement. OD600 was measured in 10mm polystyrene cuvettes with a NanoDrop™ 2000c spectrophotometer. For colony enumeration, samples were centrifuged at 8,000 × g for 2 minutes at 4°C in 1.5mL microcentrifuge tubes. After centrifugation, the cell pellet was washed, resuspended, and diluted in sterile PBS (Gibco Life Technologies, Grans Island, NY), and each dilution was plated via the drop plate method (10µL/drop) in triplicate on LB-Miller agar plates with and without selective antibiotics. Multiple replicate flasks were inoculated in tandem to ensure sufficient sample volumes for measurement and plating. Growth curves were measured from 0-4.5 h.p.i. and standard curves were generated from log phase growth samples. *S.* Typhimurium SL1344: $y = 1E+09x - 7E+08$ ($R^2=0.9833$); *S.* Typhimurium mCherry: $y = 8E+08x - 5E+08$ ($R^2=0.9579$).

For preparation of *S.* Typhimurium inocula, bacteria were grown to late log phase (3.5 hours) unless otherwise noted. At this time, two 1mL samples were taken for centrifugation and a single 1mL sample was taken for OD600 measurement. For inocula used in gentamicin protection assays, after centrifugation and resuspension in PBS,

bacteria were diluted in warm, sterile HBSS (Sigma, St. Louis, MO) to achieve the desired CFU/mL [124]. For inocula used in the streptomycin-pretreatment model of *Salmonella* colitis, 1mL samples were washed twice and resuspended in PBS to a concentration of 2×10^9 CFU/mL [47]. All inocula were plated for colony enumeration via 10µL drop plates on LB-Miller agar plates with and without selective antibiotics.

Human Intestinal Epithelial Cells

Growth and Maintenance: Wild-type T84 human colonic adenocarcinoma cells were obtained as a gift from Dr. S. Colgan, University of Colorado, Denver. Wild-type C2BBe1 cells, a clone of Caco2 human colonic adenocarcinoma cells were obtained as a gift from Dr. L. Knodler, Washington State University. Wild-type T84 cells and lentiviral-transduced T84 cells (shCD73, shNTC; see below) were maintained in T75 flasks (Corning, NY) in M4 media: Dulbecco's Modified Eagle Medium (DMEM) F-12 (Gibco, Grand Island, NY) supplemented with 10% FBS (GE Life Sciences, Logan, UT), pen-strep (Hyclone, Logan, UT), and GlutaMAX (Gibco, Grand Island, NY) until plating for final experimental format. Experimental plates included 6- or 24-well cell culture-treated plates or with 3µm inserts (Corning, NY). Wild-type C2BBe1 cells were maintained in T75 flasks in M3/transferrin media: DMEM (Gibco, Grans Island, NY) supplemented with 10% FBS, pen-strep, GlutaMAX, and human transferrin (Signma-Aldrich, St. Louis, MO) until plating in 24-well collagen-coated plates (Corning, NY). All cell incubations were performed at 37°C with 5% CO_2 and media was changed every 2-3 days. For cells used in gentamicin protection assays, cells were cultured with antibiotic-free M4 or M3/transferrin media once plated in the experimental format.

Short Hairpin RNA Lentiviral Transductions: Before the start of lentiviral transductions, puromycin kill curves were performed on wild-type T84 cells plated in a 24-well format. Increasing concentrations (0-20µg/mL) of puromycin (Sigma, St. Louis, MO) in M4 media were applied on duplicate wells for one week to determine the minimum dose needed to kill all wild-type T84 cells.

Lentiviral particles, listed in Table 1, were obtained from the Functional Genomics Facility at the University of Colorado Denver. Cells were seeded in 24-well plates and grown to 70% confluence. On the day of transduction, cells were placed in 500µL 16µg/mL polybrene in M4 media then 500µL lentiviral suspension was applied (final concentration of polybrene=8ug/mL). The next day, media was changed to M4 containing 8µg/mL puromycin. Cells were grown under puromycin selection until knockdown validation by RT-qPCR for a total of 5 passages before freezer stocks were made.

Transepithelial Electrical Resistance Readings: To ensure newly-made shRNA knockdown T84 cells were able to establish an epithelial barrier, monolayers were monitored by transepithelial electrical resistance for two weeks. Cells were seeded onto 0.33 cm^2, 3.0 µm pore size polyester Transwell™ inserts (Corning) and readings were taken using an EVOM2™ volt/ohm meter (World Precision Instruments).

Gentamicin Protection Assay

Procedure: A variety of protocol variations were used in troubleshooting this assay (see "Results") but the basic procedure used was based off of well-established laboratory protocols [99], [124]. Assays were performed the day after all IECs (T84, C2BBe1) reached 100% confluence in 6-well or 24-well plates in antibiotic-free media. After inocula was prepared as described, IEC monolayers were washed once with warm HBSS and 500µL or 250µL of *S.* Typhimurium inocula in HBSS (or HBSS alone) was added at a multiplicity of infection of 25-100. Cells were incubated for 10 minutes to allow for bacterial attachment then washed twice with warm HBSS. Cells were then placed in antibiotic-free media and incubated for another 20 minutes to allow for bacterial internalization. At this time, cells were placed in fresh media containing 50µg/mL gentamicin sulfate (RPI, Mt. Prospect, IL) and allowed to incubate until approximately 1.5 h.p.i. to kill extracellular *S.* Typhimurium. Cells were then placed in fresh media containing 5µg/mL gentamicin. For quantification of viable intracellular bacteria at various times post-infection, cells were washed with sterile PBS, solubilized in 1mL 0.2% sodium deoxycholate (Sigma, St. Louis, MO) in PBS, then diluted in PBS and plated for colony enumeration. For cells that were analyzed by qPCR, individual wells were placed in 500µL TRIzol then immediately transferred to 1.5mL microcentrifuge tubes and allowed to incubate at room temperature for at least 10 minutes before storage at -80°C. Wells containing TRIzol were washed twice with PBS before returning plates to the incubator to minimize TRIzol exposure.

Cytometric Bead Array: Cell culture supernatants collected from a gentamicin protection assay were analyzed for their expression of IL-1β, IL-8, and IL-18 using a BioLegend LEGENDplex™ Mix and Match Human Inflammation Panel (San Diego, CA) per the manufacturer's instructions. The gentamicin assay was performed as described in wild-type, shCD73, and shNTC T84 cells. For all samples, duplicate 200µL aliquots of cell culture supernatants were taken from *Salmonella*-free control wells and from wells at 1, 4, 6, 8, and 10 hours post-*S.* Typhimurium infection. Samples were immediately stored at -20°C then later thawed on ice and centrifuged at 10,000 × g for 2 minutes to remove any cell debris. The 96-well V-bottom microplate protocol was followed and samples were run on a BD LSR Fortessa Flow Cytometer and analyzed using the LEGENDplex™ Version 7.0 software.

Murine Experiments

Colony Maintenance: Specific-pathogen-free wild-type C57BL/6 and $CD73^{f/f}$ $Villin^{Cre}$ were used between 6 and 12 weeks of age for all experiments. Mice with an IEC-specific knockout of CD73 ($CD73^{f/f}$ $Villin^{Cre}$) were initially generated by breeding mice harboring two loxP sites on either side of exon 2 of the CD73 gene [4] with transgenic mice expressing Cre recombinase under control of the Villin promoter [128]. Mouse genotypes were confirmed by PCR using the CD73 loxP and $Villin^{Cre}$ primers listed in Table 2.

Once the possibility of full-body CD73 hemizygous mice in our colony became apparent, all mice were also checked for the presence of truncated CD73 alleles using the CD73 Null Allele primers listed in Table 2. Additionally, once CD73 hemizygous mice

were discovered in the colony, only homozygous $CD73^{f/f}$ $Villin^{Cre-/-}$ males and $CD73^{f/f}$ $Villin^{Cre+}$ females were used in breeding pairs.

Mice were given food and acidified water *ad libitum* unless otherwise noted. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Montana State University.

Streptomycin-Pretreatment Model of *Salmonella* Colitis: Induction of *Salmonella* colitis was performed as described in the original protocol [59]. Food and water were withdrawn from mice 4 hours prior to oral gavage with 20mg streptomycin sulfate in 75µL dH_2O. Food and water were returned until 20 hours after streptomycin treatment, when food and water were again withdrawn. After 4 hours of food and water restriction, mice were administered 1×10^8 CFU *S.* Typhimurium in 100µL PBS (or PBS alone) via oral gavage. Water was returned immediately after *S.* Typhimurium gavage and food was returned 2 hours later. Inocula were prepared as described above.

For all experiments, mouse weights were monitored daily and mice were harvested 48-72 hours post-*Salmonella* gavage. Fecal samples, mesenteric lymph nodes, liver, and spleen samples were collected post-mortem. Pellets were suspended and tissues were ground in 500uL PBS using 35mm dishes (Thermo Scientific, Rochester, NY) and the base of a 3mL syringe (BD Biosciences) before diluting and plating for colony enumeration via the drop plate method. Colon lengths were measured and IEC scrapes were collected and placed into TRIzol at this time as well.

For cohousing experiments, additional mouse fecal samples were collected before mice were sorted into their respective cages, one week after cohousing, before

streptomycin gavage, before *Salmonella* gavage, and after *Salmonella* gavage. Collected fecal samples were kept at -20°C until DNA extraction.

Mouse Colonic Epithelial Cell Scrapes: Intestinal mucosa scrapes were used to collect samples enriched in epithelial cells [145]. Full colons were isolated from mice then cut along the longitudinal axis to reveal the mucosal surface. Colons were rinsed with PBS to remove fecal matter, then a small weighing spatula was used to gently scrape along the luminal surface. Cell scrapes were then transferred to a tube containing 1mL TRIzol (Ambion, Carlsbad, CA) and dissociated by using a 1.5 inch, 18g needle and 1mL syringe.

Isolation, Identification, and Quantification or *E. faecalis* from Mice: *Enterococcus faecalis* was initially discovered on LB agar plates containing 100µg/mL streptomycin; bacteria with similar colony morphology were noted in all *Salmonella* mouse experiments. Two colonies were taken from agar plates from two $CD73^{f/f}$ $Villin^{Cre}$ mice from Mouse Experiment 3 then streaked onto LB-Miller agar without antibiotics before species identification and antimicrobial susceptibility testing by the Montana Veterinary Diagnostic Laboratory (Bozeman, MT), (Table 6, Top).

To confirm that this specific bacterium was able to be recovered from the $CD73^{f/f}$ $Villin^{Cre}$ colony after streptomycin treatment, a cage of three mice were gavaged with 20mg streptomycin. Three days later, fecal samples were collected, diluted, and plated on LB-Miller Agar containing 100µg/mL streptomycin. A colony from each mouse was then streaked onto LB-Miller agar without antibiotics before species identification and antimicrobial susceptibility testing (Table 6, Bottom). DNA extractions from mouse fecal

samples collected during Mouse Experiment 4 were performed using the DNeasy PowerSoil Kit (Quiagen). 10ng of total extracted DNA was used to perform qPCR analysis of bacterial abundance. To determine *E. faecalis* abundance in fecal samples, *E. faecalis* 16S reads were normalized to total 16S present in the sample (Table 3).

Quantification of Gene Transcripts

TRIzol reagent was used to isolate RNA from human intestinal epithelial cells or mouse colonic epithelial cell scrapes [146]. The iScript cDNA synthesis kit (Bio-Rad) was used to convert RNA transcripts into cDNA. Quantitative real-time PCR analysis was performed on a Roche LightCycler® 96 using SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method to calculate fold change and the $2^{-\Delta CT}$ method to calculate relative expression [147].

Human

Statistical Analyses

GraphPad Prism 7 (GraphPad Software, La Jolla, CA) was used to generate figures and perform statistical analyses. Probability values of less than 0.05 were considered statistically significant.

Table 1: Human shRNA Lentiviral Particles for Short Hairpin Knockdown of CD73

Target Gene	Clone	Denotation
5’-Ecto-nucleotidase, CD73 (*NT5E*)	TRCN_0000048753	shCD73 (53)
	TRCN_0000048754	shCD73 (54)
	TRCN_0000048755	shCD73 (55)
	TRCN_0000048756	shCD73 (56)
	TRCN_0000048757	shCD73 (57)
Non-Target Control	SHC016	shNTC (SS)

Table 2: Mouse Genotyping Primers

Allele	Primer Sequence
Villin-Cre [148] Transgene (Tg(Vil-cre)997Gum)	F: 5’-CATGTCCATCAGGTTCTTGC-3’ R: 5’-TTCTCCTCTAGGCTCGTCCA-3’
Internal Positive Control (oIMR7338/oIMR7339)	F: 5’-CTAGGCCACAGAATTGAAAGATCT-3’ R: 5’-GTAGGTGGAAATTCTAGATCATCC-3’
5’-Ecto-nucleotidase, CD73(*NT5E*) loxP site	F: 5’-AGAGATGTCCTTGTCATTGC-3’ R: 5’-TGGAAGTCTCCCAACTAAAA-3’
5’-Ecto-nucleotidase, CD73(*NT5E*) Null Allele	F: 5’- TCCTTGTCATTGCAACAGCC-3’ R: 5’-CTTCTGCTATGCAGCCCCTT-3
Myogenin (internal control)	F: 5’- TTACGTCCATCGTGGACAGC-3’ R: 5’- TGGGCTGGGTGTTAGCCTTA-3’

Table 3: *Enterococcus faecalis* Primers

Gene	Primer Sequence
16S rRNA	F: 5'- TCCTACGGGAGGCAGCAGT-3' R: 5'- GGACTACCAGGGTATCTAATCCTGTT-3'
16S *E. faecalis* [149]	F: 5'-CGCTTCTTTCCTCCCGAGT-3' R: 5'-GCCATGCGGCATAAACTG-3'

Table 4: Human qPCR Primers

Gene	Primer Sequence
β-actin (*ACTB*)	F: 5'- CCTGGCACCCAGCACAAT-3' R: 5'- GCCGATCCACACGGAGTAC
Heat Shock Protein 90-β (*HSP90AB1*)	F: 5'- TCTGGGTATCGGAAAGCAAGCC-3' R: 5'- GTGCACTTCCTCAGGCATCTTG-3'
5'-Ecto-nucleotidase, CD73 (*NT5E*)	F: 5'- ATTGCAAAGTGGTTCAAGT-3 R: 5'- ACACTTGGCCAGTAAAATA-3'

Table 5: Mouse qPCR Primers

Gene	Primer Sequence
β-actin (*ACTB*)	F: 5'- AACCCTAAGGCCAACCGTGAA-3' R: 5'- TCACGCACGATTTCCCTCTCA
Villin-1 (*VIL1*)	F: 5'- TCAAAGGCTCTCTCAACATCAC-3' R: 5'- AGCAGTCACCATCGAAGAAGC-3'
5'-Ecto-nucleotidase, CD73 (*NT5E*)	F: 5'- GGCAAATACCTGGGCTACCT-3' R: 5'- GTCTGCTTTGATGGTCGCAT-3'

Results and Discussion

The Gentamicin Protection Assay in shCD73 T84 Cells

Upon arriving at the facility at Montana State University, it had to be ensured that the phenotype reported by Kao et al. could successfully be recapitulated. That is, it had to be demonstrated that wild-type *S.* Typhimurium was unable to robustly replicate in shCD73 T84 cells compared to controls [3]. The criteria that was set for a successful gentamicin protection assay was that *S.* Typhimurium was able to exhibit at least 4-fold replication from 1 to 6 hours post-infection in control cells while exhibiting significantly less replication in shCD73 T84 cells [3], [99]. However, the freezer stocks of shNTC T84 cells that were brought from the facility at the University of Colorado, Denver, were relatively sparse and it took several weeks longer than the shCD73 T84 cells to propagate enough cells to perform a gentamicin protection assay.

Therefore, the gentamicin protection assay was first performed using wild-type T84 cells instead of shNTC as a positive control. These and shCD73 T84 cells were infected with wild-type *S.* Typhimurium (Figure 1). Importantly, 4-fold more bacteria were recovered at 7 h.p.i. compared to 1 h.p.i. in wild-type T84 cells, demonstrating that *S.* Typhimurium was able to properly replicate inside control cells. There was also no significant replication of *S.* Typhimurium observed in shCD73 T84 cells. Furthermore, the differences between *S.* Typhimurium replication in shCD73 and wild-type T84 cells were still detectable when CFU/mL concentrations were transformed into fold replication to normalize for bacterial entry (1 h.p.i.) (Figure 1B). Therefore, the first gentamicin

protection assay sufficiently recapitulated the finding that *S.* Typhimurium virulence was attenuated in shCD73 T84 cells compared to controls.

Once an adequate amount of shNTC T84 cells were propagated, mRNA expression levels of CD73 in wild-type, shNTC, and shCD73 T84 cells were evaluated by RT-qPCR. As was expected, CD73 expression was lower in shCD73 T84 cells compared to shNTC, and unchanged between shNTC and wild-type T84 cells (Figure 2). When the gentamicin protection assay was performed in shNTC and shCD73 cells, it was again demonstrated that *S.* Typhimurium could replicate in control cells but not in shCD73 T84 cells (Figure 3). However, there were no detectable differences in the number of bacteria recovered between 5 and 7 h.p.i. from shNTC cells and differences in fold replication were no longer statistically significant (Figure 3). Unfortunately, when the assay was repeated, there were no longer significant differences in the number bacteria recovered from shCD73 and shNTC T84 cells (Figure 4). In fact, this and every other gentamicin protection assay performed in these shNTC or wild-type T84 cells from this point forward did not meet the criteria that was set for a successful gentamicin protection assay (Figure 4, 5, 6, 7).

It was then suspected that perhaps some mutations were accrued in the shNTC T84 cells that were slow to grow in the beginning, but when shNTC T84 cells that were created in a different lentiviral-transduced short-hairpin knockdown experiment (unmatched shNTC) were used, there was still not a sufficient amount of *S.* Typhimurium recovered from infected monolayers at 7h.p.i. in control cells (Figure 5). Because there was a drift in phenotype across the four assays (Figure 1, Figure 3, Figure 4), it was

speculated that perhaps a contaminated bacterial preparation or changing the MOI of the inoculum might account for the differences across time. However, when the assay was repeated in shCD73, shNTC, and wild-type T84 cells with a different freezer stock vial of *S.* Typhimurium, freshly-made LB broth and LB agar plates, and applied the bacteria at an MOI of 30, the assay was still unsuccessful (Figure 6).

At this point, troubleshooting experiments were performed by addressing various protocol variations in the gentamicin protection assay that might account for the inability of *S.* Typhimurium to replicate in T84 cells. For cells used in the Kao et al. paper, T84 cells were maintained in media containing Pen-Strep until 24 hours before the starts of the gentamicin protection assay, cells were previously maintained in antibiotic-free media once they were plated in their experimental format (personal communications). However, when wild-type and shCD73 T84 cells to were used compare differences in these cell culture techniques, the assay was still unsuccessful (data not shown). Also, T84 monolayers change their gene expression with monolayer maturation after becoming 100% confluent, so day old, week old, and two-week-old monolayers were tested, but *S.* Typhimurium appeared to be unable to replicate in any instance (data not shown) [150].

It was also possible that *S.* Typhimurium was replicating in the T84 cells but there was another element to the methods that were being used that was masking bacterial growth. To address this concern, several more assays were performed in which different concentrations of gentamicin were applied in the media and composition of the solubilization buffer used to lyse T84 cells was changed. In one assay, higher concentrations of gentamicin were used in the media to kill off the extracellular bacteria.

Whereas in most assays 50µg/mL gentamicin was used in the media for about an hour and 5µg/mL was used for the remainder of the assay, 100µg/mL and 10µg/mL ere applied, respectively, but there were no noted difference in recovered bacteria (data not shown) [99]. A shorter, 30-minute application of 50µg/mL of gentamicin was also tried, but there was also no difference in the amount of *Salmonella* recovered over time (data not shown) [126]. The use of 1% Triton X-100 and 0.1% sodium dodecyl sulfate solubilization buffer was also unsuccessful (data not shown) [3].

Finally, *S.* Typhimurium was grown to mid-log phase instead of late-log phase, but there were no differences in the number of bacteria recovered (data not shown, Figure 7) [3]. Interestingly, when the assays were performed using bacteria grown to mid-log phase instead of late-log phase, there were no measured differences in bacterial invasion as measured by the number of bacteria recovered at 1 h.p.i. This observation is notable because the growth phase of *S.* Typhimurium used for gentamicin protection assay inocula has been shown to be critically important for invasion and intracellular replication (Figure 6, Figure 7) [125]. Throughout all of this troubleshooting, 6-well and 24-well plates, new antibiotic stocks and reagents, and the frequency of cell media changes were altered (data not shown), but none of these protocol changes made a difference in the success of the gentamicin protection assay. At this point in time, both the wild-type and short hairpin knockdown T84 cells were at a high passage number and other researchers in the lab had noted a drift in the gene expression of their targets of interest in the wild-type cells. Also, it was still possible that something in the preparation of inocula or other reagents was preventing robust *S.* Typhimurium replication in T84

cells, so new shCD73 and shNTCs were made from a fresh freezer stock of wild-type T84 cells to continue troubleshooting the gentamicin protection assay.

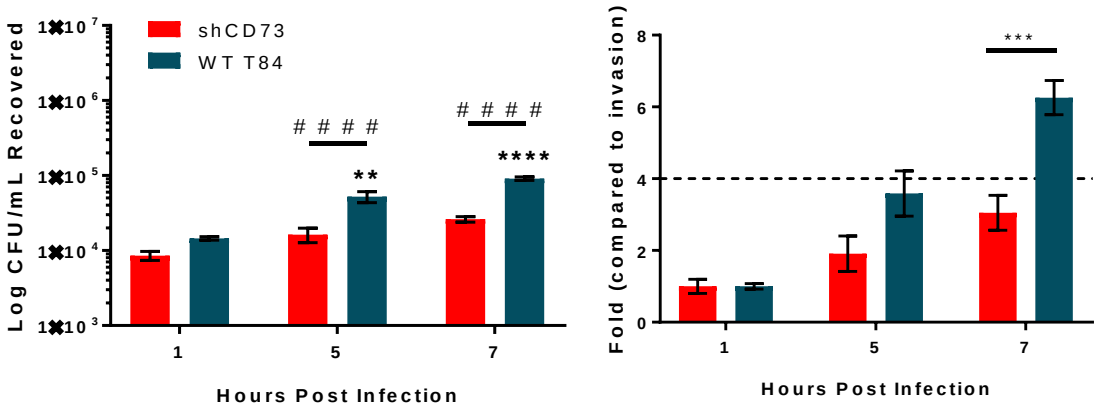

Figure 1. Gentamicin Protection Assay: shCD73 T84 v. Wild-type T84 Cells, MOI=30 *S.* Typhimurium SL1344. (A) Analyzed by two-way repeated measures ANOVA with Dunnett's multiple comparisons test. **** denotes p<0.0001 from 1 h.p.i. using Dunnett's multiple comparisons test; # denotes p<0.0001 between groups using Sidak's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. Analyzed by two-way ANOVA with Sidak's multiple comparisons test. *** denotes p<0.001. N=3 wells of 6-well plate.

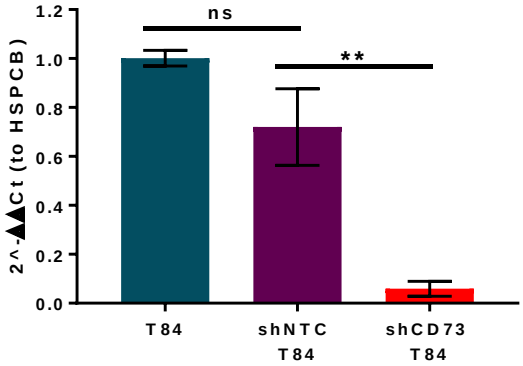

Figure 2. Baseline Fold Change in CD73 mRNA Expression in Wild Type and Short Hairpin Knockdown T84 Cells. Normalized to *HSPCB.* Analyzed via one way ANOVA with Tukey's multiple comparisons test. ** denotes p<0.01. N=6

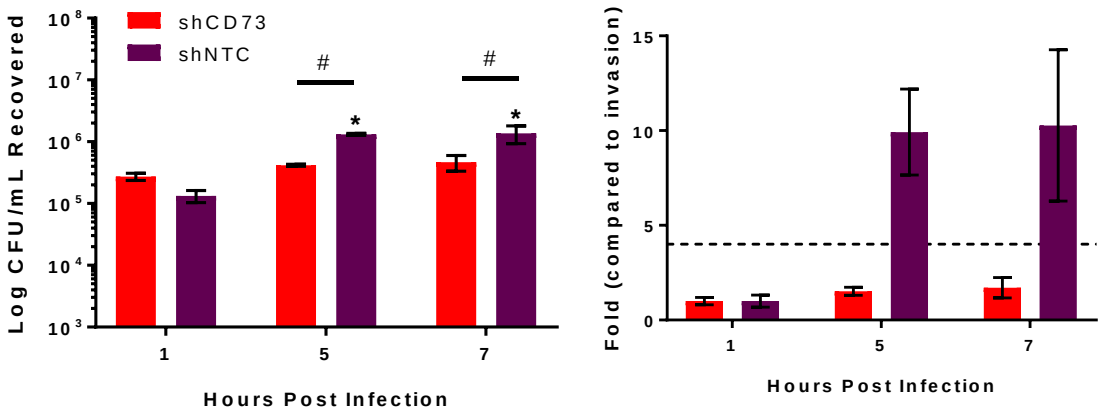

Figure 3. Gentamicin Protection Assay: shCD73 v. shNTC T84 Cells, MOI=130 *S.* Typhimurium SL1344. (A) * denotes $p<0.05$ from 1 h.p.i. using Dunnett's multiple comparisons test; # denotes $p<0.05$ between groups using Sidak's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=2 wells of a 6-well plate.

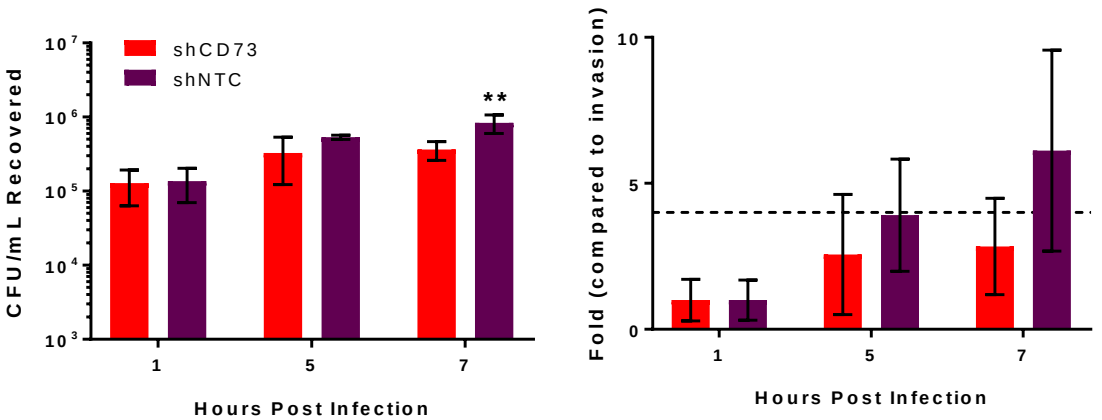

Figure 4. Gentamicin Protection Assay: shCD73 v. shNTC T84 Cells, MOI=130 *S.* Typhimurium SL1344. (A) Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. ** denotes $p<0.01$ from 1 h.p.i. using Dunnett's multiple comparisons test (B) Dashed line represents defined replication threshold for valid assay. N=2 wells of 6-well plate.

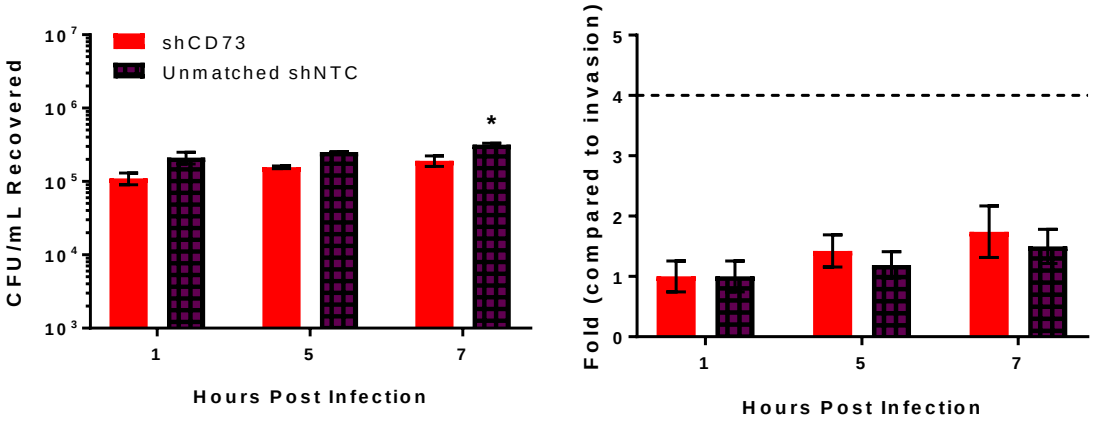

Figure 5. Gentamicin Protection Assay: shCD73 T84 v. unmatched shNTC, MOI=90 *S.* Typhimurium SL1344. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. * denotes $p<0.01$ from 1 h.p.i. using Dunnett's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=2 wells of a 6-well plate

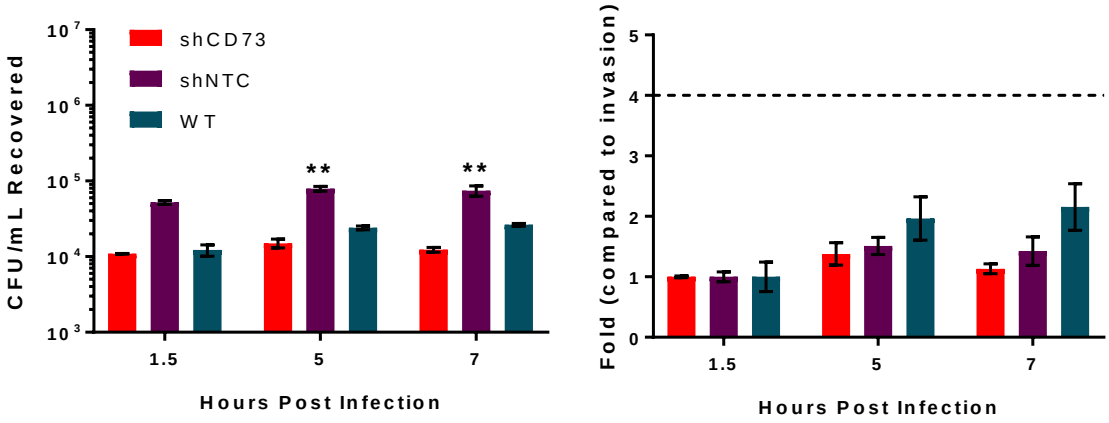

Figure 6. Gentamicin Protection Assay: shCD73, shNTC and wild-type T84 Cells, MOI=30 *S.* Typhimurium SL1344. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. ** denotes $p<0.01$ from 1 h.p.i. using Dunnett's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=3 wells of a 24-well plate.

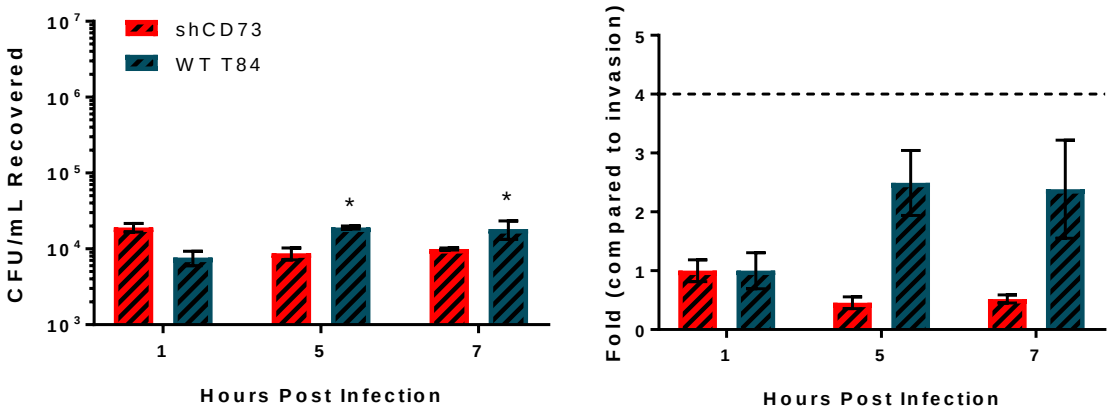

Figure 7. Gentamicin Protection Assay: shCD73 and wild-type T84 Cells, MOI=25 *S.* Typhimurium SL1344 Grown to Mid-Log Phase. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. * denotes $p<0.05$ from 1 h.p.i. using Dunnett's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=3 wells of a 24-well plate.

Remaking and Testing New shCD73
Knockdown T84 Cells

Low-passage wild-type T84 cells were transduced with five different lentiviral particle preparations containing plasmids encoding shRNA targeted toward CD73 transcripts and one non-target shRNA lentivirus control (Table 1). Once cells were maintained in puromycin selective media for several passages and all non-lentiviral transduced control cells had died, CD73 mRNA expression was checked to identify successful shCD73 candidates (Figure 8). To ensure these cells could form confluent monolayers and establish an epithelial barrier, cells were plated on transwell inserts and monitored transepithelial electrical resistance (TEER) over time (Figure 9). TEER was monitored in these cells because Kao et al. had never noted any defects in barrier formation in the original shCD73 T84 cells (personal communications). The shCD73 constructs 54 and 55 were used to proceed with testing since they had the lowest

expression of CD73 and there were no consistent differences in TEER between these cells and the new shNTC T84 cells (shNTC SS).

Starting with a different freezer stock of SL1344 from Dr. Leigh Knodler, Washington State University (SL1344W), these newly-made shRNA T84 cells were used in a gentamicin protection assay. Like previous findings, there was no observed growth of *S.* Typhimurium in shNTC SS cells and no differences between cell types (Figure 10). However, it was noted that there was a measured difference in CD73 expression between wild-type and shNTC SS T84 cells (Figure 8B) so wild-type cells were included in the next assays. However, no matter whether *S.* Typhimurium was grown to late-log phase or mid-log phase, there was still no measured intracellular growth in T84 cells over time (data not shown, Figure 11). Finally, the possibility that a mistake had been made in the creation or maintenance of the bacterial freezer stocks could not be ruled out, so new *S.* Typhimurium SL1344 mCherry stab cultures were sent from Dr. Kao and new bacterial freezer stocks were prepared. Still, no intracellular bacterial replication in wild-type T84 cells was recorded using newly-prepared *S.* Typhimurium (Figure 12).

Even though the role of CD73 could not be investigated using bacterial enumeration in the gentamicin protection assay, it was possible that differences could be detected in cytokine levels that may be affected by the absence of CD73 throughout the assay. Therefore, a multiplex cytokine bead array was performed that allowed for the measurement of IL-8, IL-18, and IL-1β concentrations simultaneously from the supernatants collected throughout a gentamicin protection assay with wild-type, shNTC SS, and shCD73 (55) T84 cells. IL-8 was a cytokine of interest since IECs secrete IL-8

in response to *Salmonella* entry and it has been shown that IL-8 secretion is upregulated when extracellular purine nucleotide metabolism is disrupted [106], [151]–[153]. Therefore, it was hypothesized that IL-8 secretion may also be upregulated in shCD73 T84 cells. IL-1β and IL-18 were cytokines of interest because they are associated with inflammasome activation in response to *S.* Typhimurium and high concentrations of extracellular ATP [90], [114], [117]. It is unknown if the absence of CD73 impacts extracellular ATP concentrations in the intestinal epithelium, but we speculated that extracellular ATP may be upregulated in the absence of CD73 to metabolize AMP. Therefore, it was hypothesized that IL-18 and IL-1β would also be upregulated in shCD73 cells. IL-1β measurements at or below the limit of detection of 1.6 pg/mL for all cell types at all time points, also CD73 had no effect on IL-18 secretion at these timepoints (data not shown). However, at 10 h.p.i. *S.* Typhimurium infection, shCD73 T84 cells produced more IL-8 than wild-type or shCD73 T84 cells (Figure 13). This finding suggests that CD73 may be important in modulating IL-8 secretion from IECs in response to *S.* Typhimurium. It also confirms that the shCD73 T84 cells respond differently to infection with *S.* Typhimurium than control cells, even if there were no differences between cell types in bacterial replication.

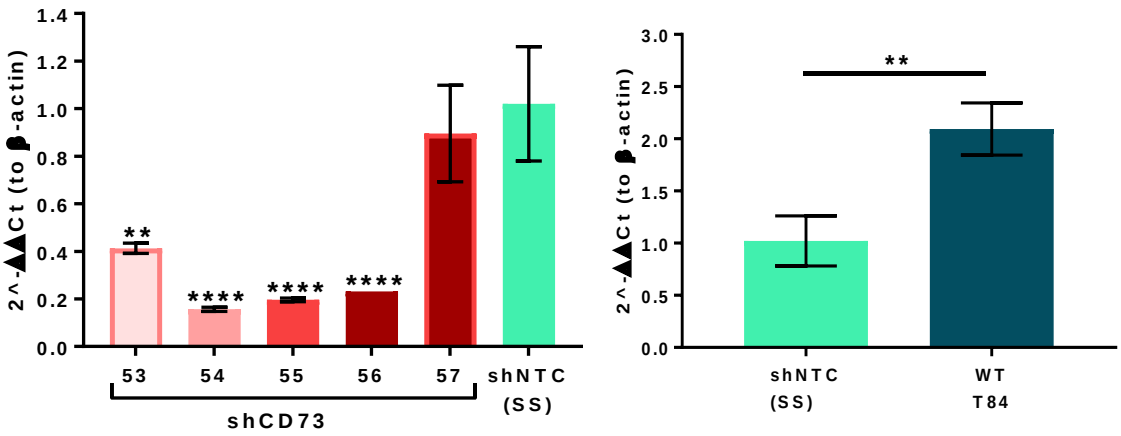

Figure 8. Baseline Fold Change in CD73 mRNA Expression in New Short Hairpin Knockdown T84 Cells. (A) Analyzed via one-way ANOVA with Tukey's multiple comparisons. ** denotes p<0.01, **** denotes 0.0001 compared to shNTC. (B) New shNTC T84s compared to wild-type T84 cells. Analyzed via unpaired t-test. N=3 wells of a 24-well plate.

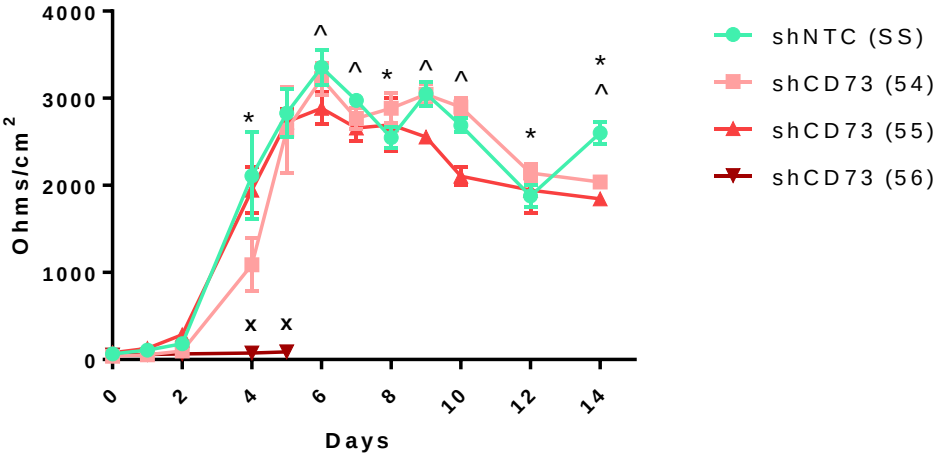

Figure 9. Transepithelial Electrical Resistance Readings of Short Hairpin Knockdown T84 Cells. Symbols (* = 54; ^ = 55; x = 56) denote p < 0.05 compared to shNTC. N=6 transwell inserts.

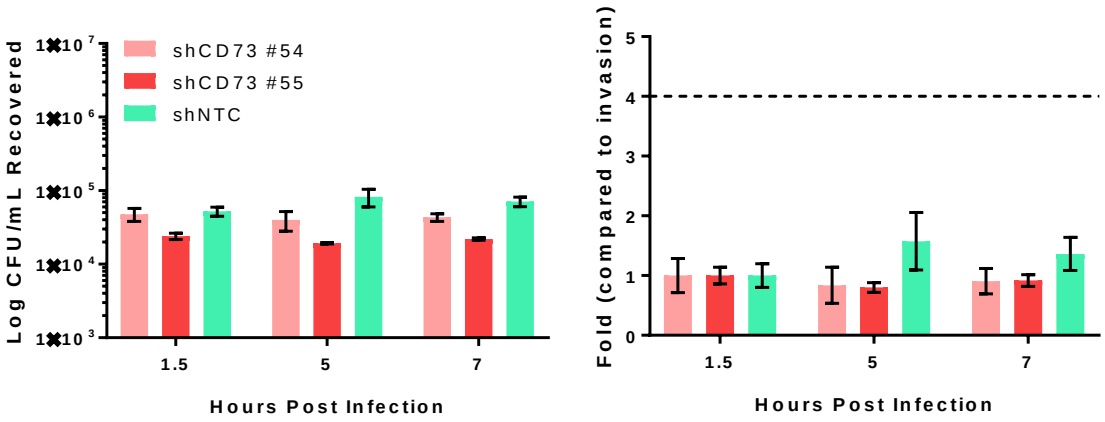

Figure 10. Gentamicin Protection Assay: New shCD73, shNTC and wild-type T84 Cells, MOI=135 *S.* Typhimurium SL1344, with Higher Gentamicin Concentrations. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=3 wells of a 24-well plate.

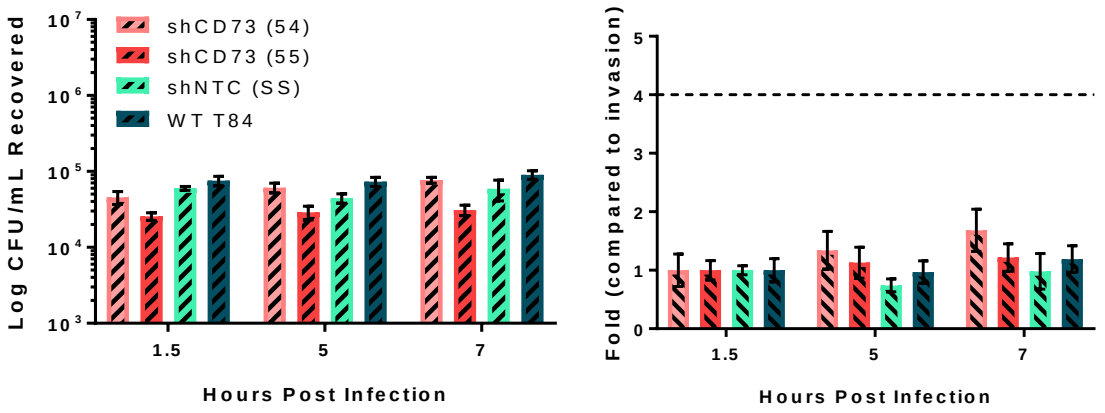

Figure 11. Gentamicin Protection Assay: New shCD73, shNTC and wild-type T84 Cells, MOI=30 *S.* Typhimurium SL1344 Grown to Mid-Log Phase. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=3 wells of a 24-well plate.

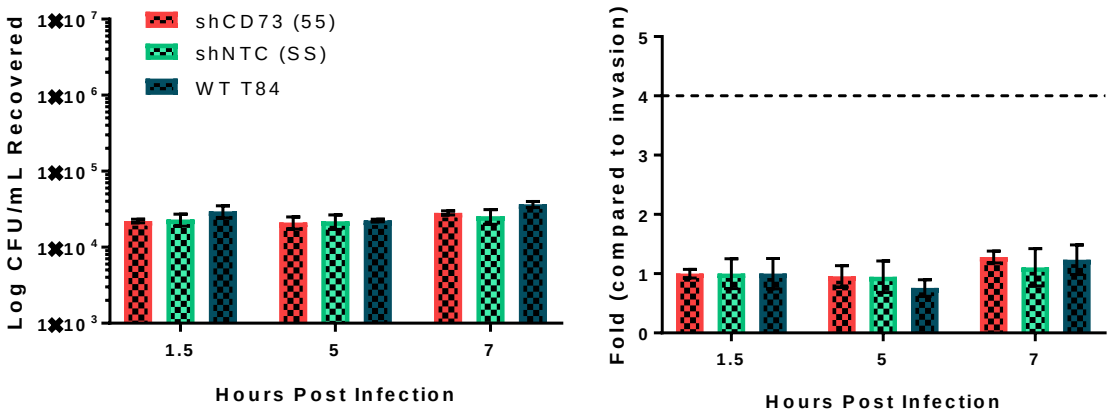

Figure 12. Gentamicin Protection Assay: New shCD73, shNTC and wild-type T84 Cells, MOI=30 *S.* Typhimurium SL1344 mCherry. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=3 wells of a 24-well plate.

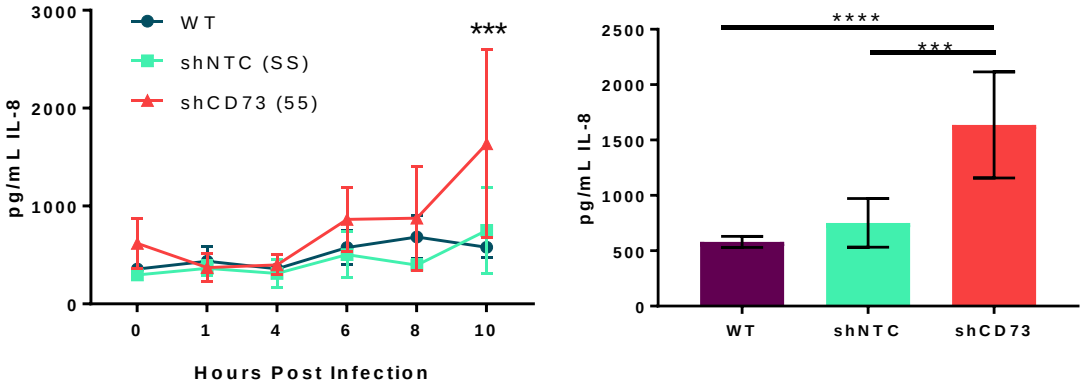

Figure 13. Cytometric Bead Array Analysis for IL-8 Recovered from Wild-type and New Short Hairpin Knockdown T84 Cells During a Gentamicin Protection Assay. Analyzed via two-way ANOVA with Tukey's multiple comparisons test. *** denotes $p<0.001$, **** denotes $p<0.0001$, compared to shCD73 T84 cells.

Testing New Human Intestinal Epithelial Cell Lines

When the gentamicin protection assay had been attempted in T84 cells with a multitude of protocol variations and was unsuccessful in control cells, a different human colonic intestinal epithelial cell line, Caco-2 BBe1 (C2BBe1), was used in a gentamicin protection assay. The assay was attempted three separate times with *S.* Typhimurium SL1344W at an MOI of 75 and each time bacterial replication over time was measured in C2BBe1 cells, but never measured in wild-type T84 cells (Figure 14). The ability to perform successful gentamicin protection assays in C2BBe1 cells but not in T84 cells suggests this project may require a new *in vitro* intestinal epithelial cell culture model to investigate the role of CD73 in *S.* Typhimurium pathogenesis.

When CD73 mRNA expression was measured, it was found that C2BBe1 cells express very little CD73 at baseline (Figure 15). Therefore, they would not be a suitable alternative to T84 cells for performing CD73 shRNA knockdowns, but lentiviral particles containing a CD73 open reading frame construct could be used to create a C2BBe1 cell line that over-expresses CD73. However, HT-29 human intestinal epithelial cells appear to have similar CD73 expression patterns as T84 cells at baseline (Figure 15). Although these cells were not tested in a gentamicin protection assay, they are a common cell line used for *S.* Typhimurium studies, so they might represent a good alternative to using T84 cells to create a shCD73 cell line.

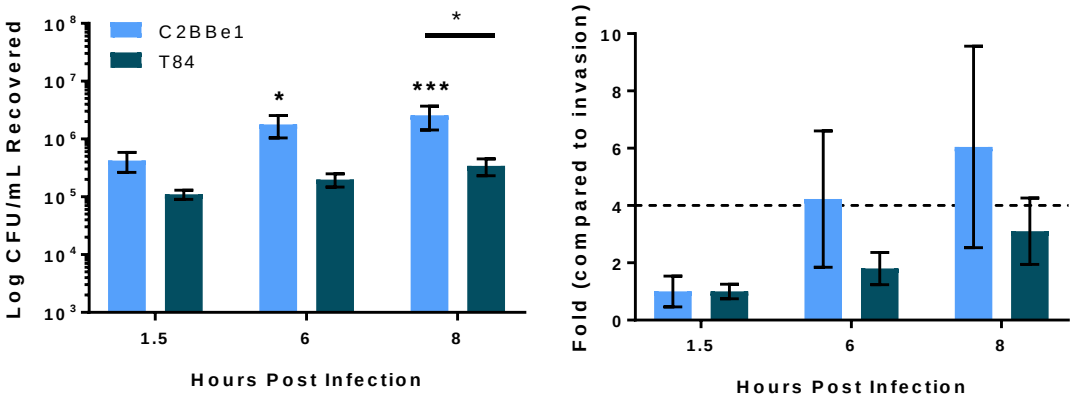

Figure 14. Gentamicin Protection Assay: Wild-type C2BBe1 and T84 Cells, MOI=75 *S.* Typhimurium SL1344. Analyzed with two-way repeated measures ANOVA with Dunnett's multiple comparisons and Sidak's multiple comparisons test. (B) Dashed line represents defined replication threshold for valid assay. N=9 total wells from 3 separate assays performed in 24-well plates.

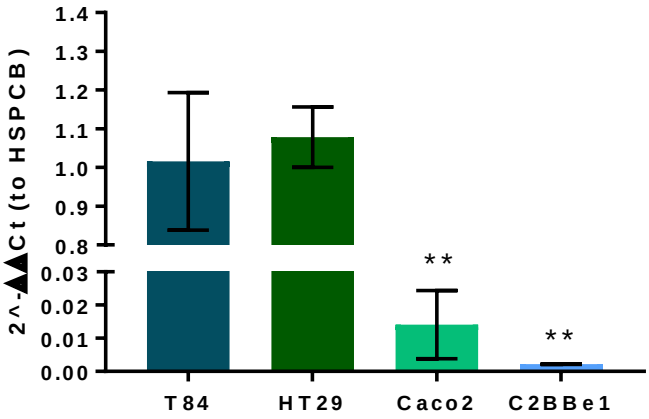

Figure 15. Baseline Fold Change in CD73 mRNA Expression Wild-type T84, HT-29, Caco-2, and C2BBe1 Human Intestinal Epithelial Cell Lines. ** denotes $p<0.01$ compared to T84. N=2 wells of a 6-well plate.

Streptomycin-Pretreatment Model of *Salmonella* Colitis

Previous work from our lab had shown that CD73 in the intestinal epithelium modulates *S.* Typhimurium bacterial load in intestinal contents and influences dissemination to distal sites in the streptomycin-pretreatment model of *Salmonella* colitis [3]. To test whether this finding was repeatable at in the facility at Montana State University, $CD73^{f/f}$ $Villin^{Cre-/-}$ (Cre (-)) and $CD73^{f/f}$ $Villin^{Cre+}$ (Cre (+)) mice were pretreated with streptomycin then gavaged with wild-type *S.* Typhimurium SL1344 mCherry or PBS. Infection was allowed to progress for 72 hours before sacrifice.

It was expected that more *Salmonella* would be recovered from the intestinal contents and fewer *Salmonella* would be recovered from distal sites (mesenteric lymph nodes, liver, spleen) in Cre (+) mice due to the lack of CD73 in their intestinal epithelium, but no differences were found between the bacterial load in Cre (+) and Cre (-) mice (Figure 16). It was also expected that Cre (+) mice would have longer colons compared to Cre (-) mice since it was previously found that mice lacking CD73 in their IECs were protected from *Salmonella* colitis-induced colon shortening compared to littermate controls, but again, this finding was not repeated here (Figure 16). Still, colon lengths were shorter after 72 hours of *Salmonella* exposure compared to colons from streptomycin-treated control mice (Figure 16) and *Salmonella*-exposed mice did exhibit weight loss over time following *Salmonella* gavage (Figure 17). Additionally, gross intestinal pathology was apparent in *Salmonella*-treated mice compared to controls, which was exemplified by the inability to recover any colon contents from three $CD73^{f/f}$ $Villin^{Cre}$ mice. This information taken with the fact that *Salmonella* was recovered from distal sites suggested that the streptomycin-pretreatment model was working as expected,

but perhaps that disease had progressed too far to catch any subtle difference in *S.* Typhimurium virulence between Cre (+) and Cre (-) mice. Furthermore, the *S.* Typhimurium SL1344 mCherry strain had not been very well-characterized *in vitro* so it was possible that something was wrong with the inoculum itself. However, because there were no noted differences between Cre (+) and Cre (-) mice, genotyping results needed to be evaluated in the intestinal epithelium as well.

Although in the mouse genotyping scheme had included assays to check for the presence of $Villin^{Cre}$ and a loxP site on one side of exon 2 in mouse DNA from mouse tail snips (Table 2), Cre recombinase activity or resultant *CD73* expression in the intestinal epithelium had not been checked after the mice arrived at the MSU facility. Therefore, the expression of CD73 mRNA was checked in colonic epithelial scrapes in *Salmonella*-exposed and naïve $CD73^{f/f}$ $Villin^{Cre}$ mice to confirm that Cre (+) mice had diminished expression of CD73 compared to Cre (-) mice. Two naïve wild-type mice were also included in this experiment, since there should be minimal differences between Cre (-) and wild-type mice if the conditional knockout technology was working as expected. Unfortunately, the results demonstrated that there were no significant differences in CD73 mRNA expression between Cre (+) and Cre (-) mice either before or after *Salmonella* exposure, but that all $CD73^{f/f}$ $Villin^{Cre}$ mice expressed significantly less CD73 in IECs compared to wild-type mice (Figure 18A). Conversely, when Cre (+) and Cre (-) mouse IEC samples were combined by *S.* Typhimurium exposure for analysis, it was found that *Salmonella*-infected mice expressed less CD73 than naive mice (18B). This

finding is in agreement with previous reports demonstrating that CD73 expression is downregulated in response to *S.* Typhimurium infection in mice [2].

The lack of any difference in CD73 expression in the epithelium of Cre (+) and Cre (-) mice suggested the mouse line was potentially exhibiting leaky Cre recombinase expression. It has previously been shown that conditional knockout mice harboring a Cre recombinase gene under the control of tissue-specific gene promoters can sometimes express Cre recombinase in the testes in addition to their typical tissue site. Cre expression in the testes can lead to a full genome knockout of floxed alleles in sperm, leaving progeny with a total body null allele [154]. After further investigating the $Villin^{Cre}$ mouse originally used in generating the $CD73^{f/f}$ $Villin^{Cre}$ mouse line, it was discovered that leaky Cre in the testes was documented for this mouse [128]. This meant that any breeding pair in which a male Cre (+) mouse was used, resultant pups could be whole body CD73 null, regardless of Cre recombinase expression. It became apparent that Cre (+) male mice had been used in breeding pairs, but since there was an entire litter that was of age and there was not time to develop an assay to detect null alleles before they were too old, a group of wild-type mice were used for reference and the streptomycin-pretreatment experiment was repeated.

Taking into consideration the possible variables that could have impacted the success of the first experiment, in the second streptomycin-pretreatment mouse experiment, the following protocol changes were made: wild-type control and *Salmonella*-infected groups were included, mice were infected with the plasmid-free wild-type *S.* Typhimurium SL1344 strain that was used for *in vitro* experiments, infection

was only allowed to progress for 48 hours, and cecal contents were collected for enumeration of intestinal bacteria load instead of colon contents. Nonetheless, although experimental parameters were shifted, the results of this second streptomycin-pretreatment experiment were comparable to the first *Salmonella* mouse experiment, as there were no significant differences discovered between any groups in bacterial load, weight loss, or colon shortening (Figure 19, Figure 20, Figure 21). It is important to note that in this experiment, the start day of wild-type mice was staggered by one day, so they received a different inocula preparation, although they were prepared in the same manner. Meanwhile, a researcher in the lab had developed an assay to detect mice that were CD73 null and checked for appropriate gene recombination in the intestinal epithelium, so this restored confidence in the mouse genetics of the $CD73^{f/f}$ $Villin^{Cre}$ mice (Table 2) (H. Grifka-Walk, unpublished data).

Since the mouse genotypes in the previous two experiments was uncertain, wild-type mice were exposed to streptomycin and *Salmonella* on a different day than $CD73^{f/f}$ $Villin^{Cre}$ mice, and the first two experiments did not have completely sex-matched groups, the streptomycin-pretreatment experiment was repeated again with *S.* Typhimurium SL1344 mCherry in female wild-type and $CD73^{f/f}$ $Villin^{Cre}$ mice. The mCherry strain was chosen again since there were issues with the plasmid-free SL1344 strain in the gentamicin protection assay and the mCherry strain was the exact isolate used by Kao et al. [3] to designate a role for CD73 in the intestinal epithelium. In this third *Salmonella* experiment, mice were again harvested after only 48 hours post-infection and no significant differences were found between Cre (+) and Cre (-) mice by

any metric (Figure 22, Figure 23, Figure 24). However, while bacterial load and weight loss did not significantly differ between Cre (+) and Cre (-) mice, wild-type mice exhibited shorter colons than $CD73^{f/f}$ $Villin^{Cre}$ mice after *Salmonella* exposure (Figure 23).

One notable observation that was made in all three experiments that was originally overlooked was the appearance of small, white colonies that were distinct from the large, pink or white *S.* Typhimurium colonies on agar plates used for quantification of bacterial load. Although inocula were always plated on both plain LB agar and LB agar containing100ug/mL streptomycin, only streptomycin plates were used for quantification of murine fecal or tissue samples. The small, streptomycin-resistant colonies were found primarily on plates corresponding to $CD73^{f/f}$ $Villin^{Cre}$ mouse samples.

In the first experiment, these small colonies were only found in streptomycin-treated control fecal samples, but not in any *Salmonella*-exposed mouse samples. In the second experiment, they were again found on $CD73^{f/f}$ $Villin^{Cre}$ streptomycin-treated control fecal samples but not in any wild-type mouse samples. Small colonies were also found in several *Salmonella*-exposed mouse samples, although it was not noted which specific samples contained these small colonies. In the third experiment, small colonies were found in every $CD73^{f/f}$ $Villin^{Cre}$ streptomycin-treated control fecal sample but there were also two small colonies found in a single wild-type streptomycin-pretreated control mouse fecal sample. It was also noted that small colonies were found on several *Salmonella*-exposed $CD73^{f/f}$ $Villin^{Cre}$ mouse sample plates, but this time they were found both in fecal samples and in mesenteric lymph nodes.

It was becoming clear that this small colony could be consistently isolated from CD73$^{f/f}$ Villin^{Cre} mice after gavage with streptomycin, and much less frequently in wild-type mice. Therefore, since the genetics of the CD73$^{f/f}$ Villin^{Cre} mice had been confirmed, and since this small colony bacterium was never noted in experiments by Kao (personal communications) it was suspected that the small colony, streptomycin-resistant bacterium may be interfering with the streptomycin-pretreatment model itself in CD73$^{f/f}$ Villin^{Cre} mice. It became reasonable to believe that the small colony bacterium may represent a member of the CD73$^{f/f}$ Villin^{Cre} commensal microbiome that interferes with the competitive advantage given to *Salmonella* by streptomycin pretreatment. However, other possibilities could not be ruled out, such as contaminating agents that were present only in the CD73$^{f/f}$ Villin^{Cre} colony, so two colonies were chosen from the third mouse experiment to submit for species identification and antimicrobial susceptibility testing (Table 6, top). Additionally, colonies with similar morphology were isolated from the feces of three more CD73$^{f/f}$ Villin^{Cre} mice in the colony after gavage with streptomycin and were submitted for identification as well (Table 6, bottom).

All unknown isolates were identified as *Enterococcus faecalis*, a Gram-positive bacterium that is commonly isolated from the human and mouse gastrointestinal tract. *E. faecalis* is known both as an opportunistic pathogen and a potential probiotic species with several studies indicating its ability to confer protection to its animal host in the face of more virulent pathogens [155]–[158]. Most importantly, Castro et al. showed that *E. faecalis* was protective of *Salmonella* Enteritidis infection which is supportive of the

hypothesis that *E. faecalis* might be protective of *Salmonella* colitis in experiments using serovar Typhimurium the streptomycin-pretreatment model [156], [157].

To investigate the influence of *E. faecalis* on the streptomycin-pretreatment model further, an experiment was performed in which wild-type and $CD73^{f/f}$ $Villin^{Cre}$ mice were housed in separate cages or housed together for two weeks in order to normalize the intestinal microbiota between the colonies [159], [160]. These mice were then used in a fourth streptomycin-pretreatment experiment using *S.* Typhimurium mCherry. While no differences were found between any groups in *Salmonella* bacterial load or colon length (Figure 25), separately-housed $CD73^{f/f}$ $Villin^{Cre}$ mice appeared to be protected from *Salmonella*-induced weight loss compared to wild-type or cohoused animals on days 1 and 2 post-*S.* Typhimurium gavage (Figure 26).

Mouse fecal samples were also collected before cohousing (day -14), one week after cohousing (day -7), before streptomycin gavage (day-1), after streptomycin gavage (day 0), and one day-post *Salmonella* gavage (day 1). Two mice were then chosen from each group to track *E. faecalis* abundance in feces over time. From these fecal samples, DNA was extracted and relative abundance of *E. faecalis* in mouse feces was quantified by normalizing *E. faecalis* 16S reads to total bacterial 16S detected in each sample. *E. faecalis* was only detected in one wild-type mouse before streptomycin gavage, but was detected in three of the four $CD73^{f/f}$ $Villin^{Cre}$ mice before exposure to streptomycin (Figure 27). These results taken with the experimental transcripts noting that bacteria with similar characteristics appear in higher frequency in $CD73^{f/f}$ $Villin^{Cre}$ mouse sample suggest that *E. faecalis* might be present in lower abundance in the microbiome of wild-

type mice compared to mice from the CD73$^{f/f}$ Villin^{Cre} colony. Additionally, *E. faecalis* relative abundance increased in all analyzed samples after streptomycin gavage and either increased or stayed the same after *S.* Typhimurium exposure (Figure 27). Since it has been demonstrated that *E. faecalis* can impart host protection against *Salmonella* infection, these results provide evidence for the hypothesis that *E. faecalis* may be acting as a confounding variable in the experiments using the streptomycin-pretreatment model of *Salmonella* colitis [156].

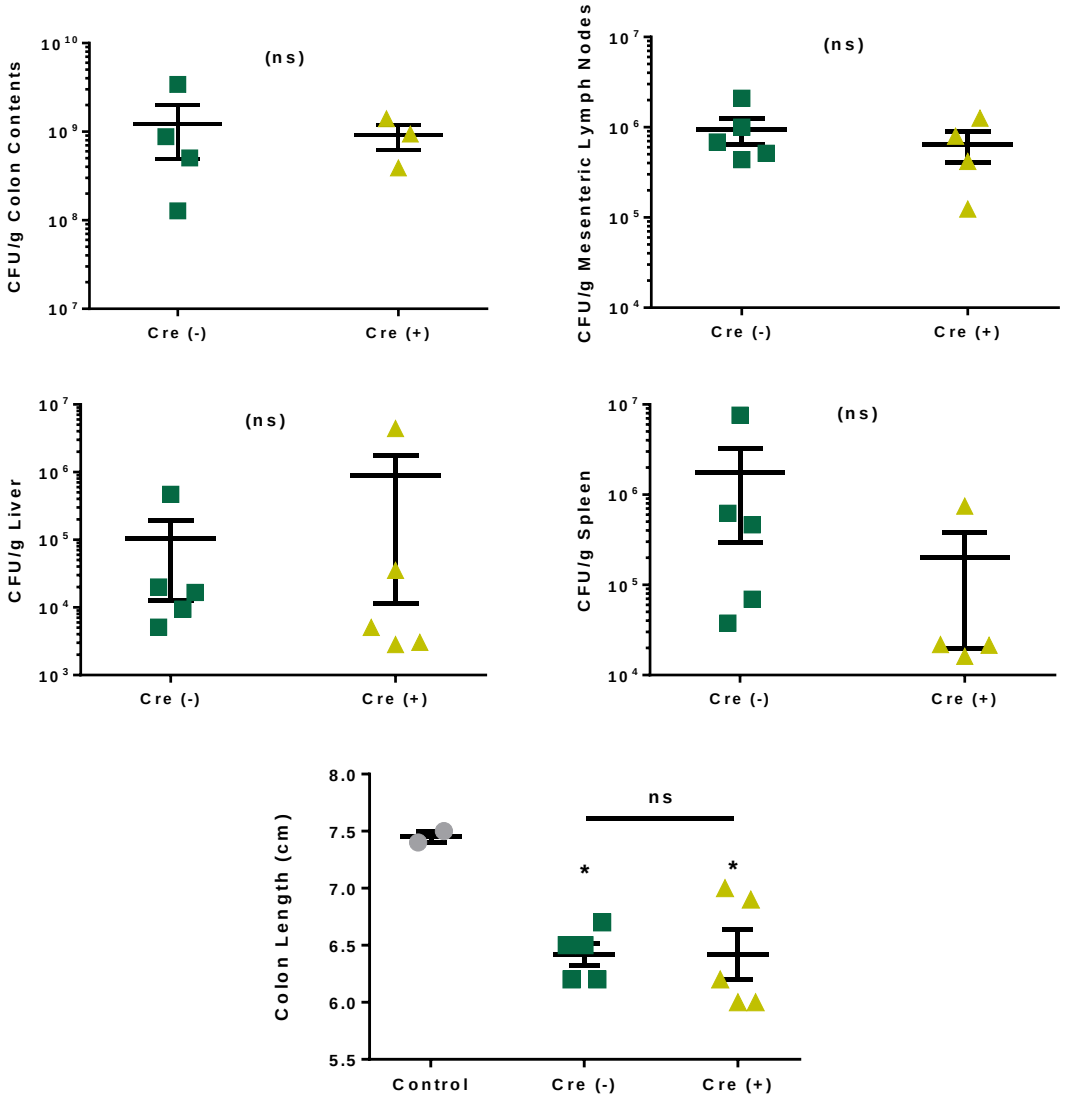

Figure 16. Mouse Experiment 1: Colon Length and Bacterial Load in Mouse Colon Contents and Tissues 72 Hours Post-Infection with *S.* Typhimurium mCherry. Symbols represent individual mice. Analyzed with an unpaired t-test.

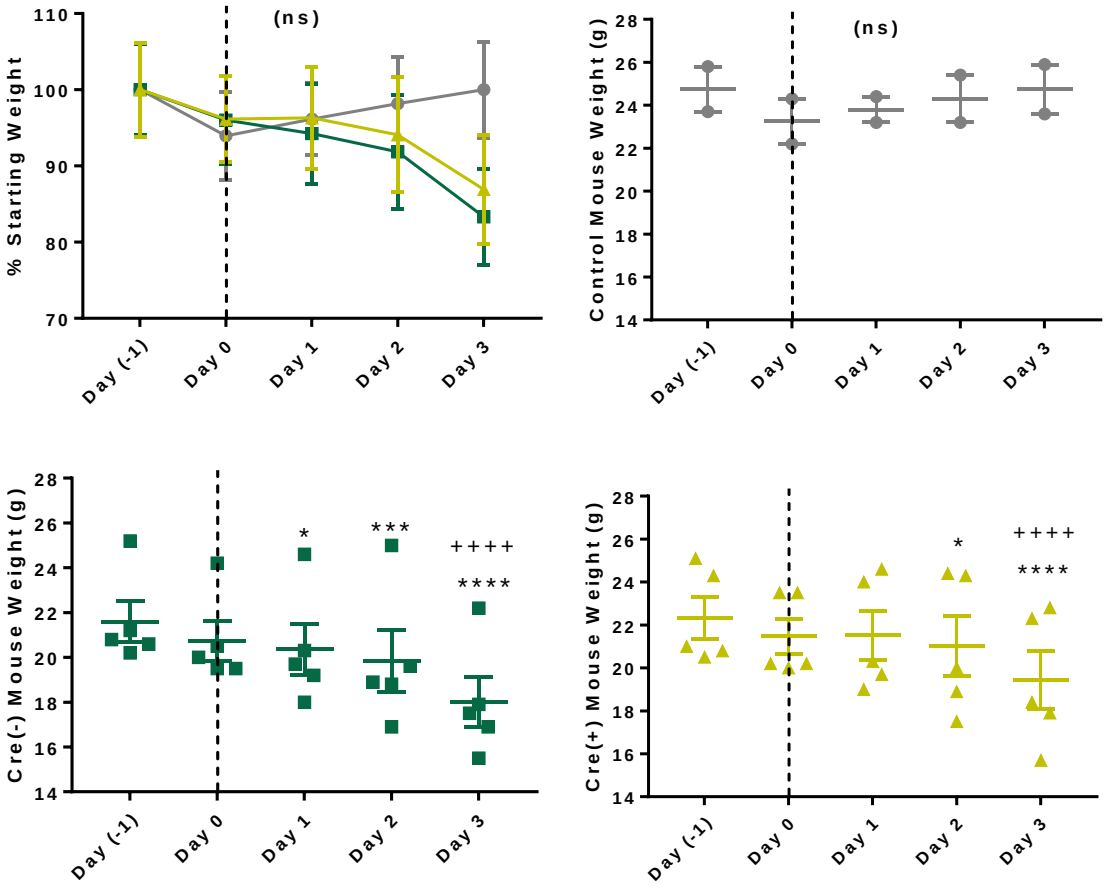

Figure 17. Mouse Experiment 1: Mouse Weight Over Time Through 72 Hour Infection with *S.* Typhimurium mCherry. Analyzed via two-way ANOVA with Tukey's multiple comparisons tests. * denotes difference from day -1 (before streptomycin gavage), + denotes difference from day 0 (before *Salmonella* gavage).

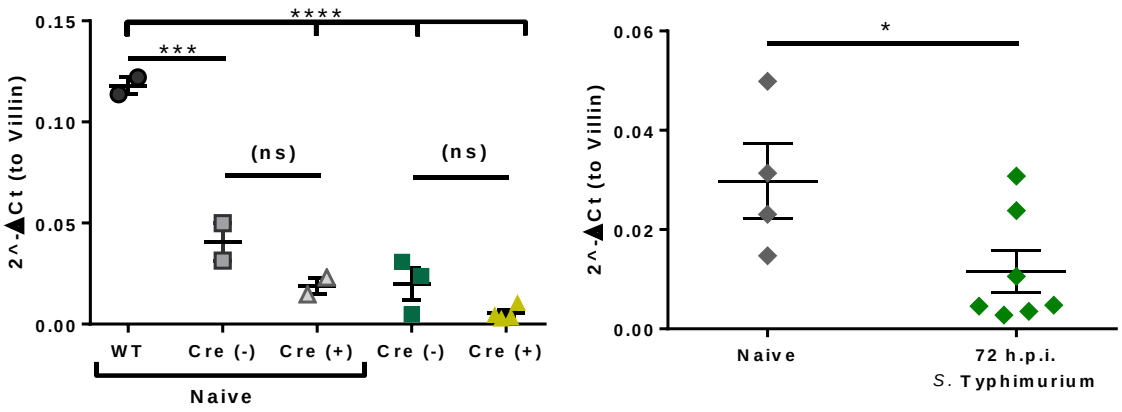

Figure 18. Relative Expression *CD73* mRNA in Mouse Intestinal Epithelial Cell Scrapes from Naive and *Salmonella*-exposed Mice. Symbols represent individual mice. (A) Analyzed by one-way ANOVA with Tukey's multiple comparisons test. *** denotes $p<0.01$, **** denotes $p<0.0001$ (B) Analyzed by unpaired t-test. * denotes $p<0.05$.

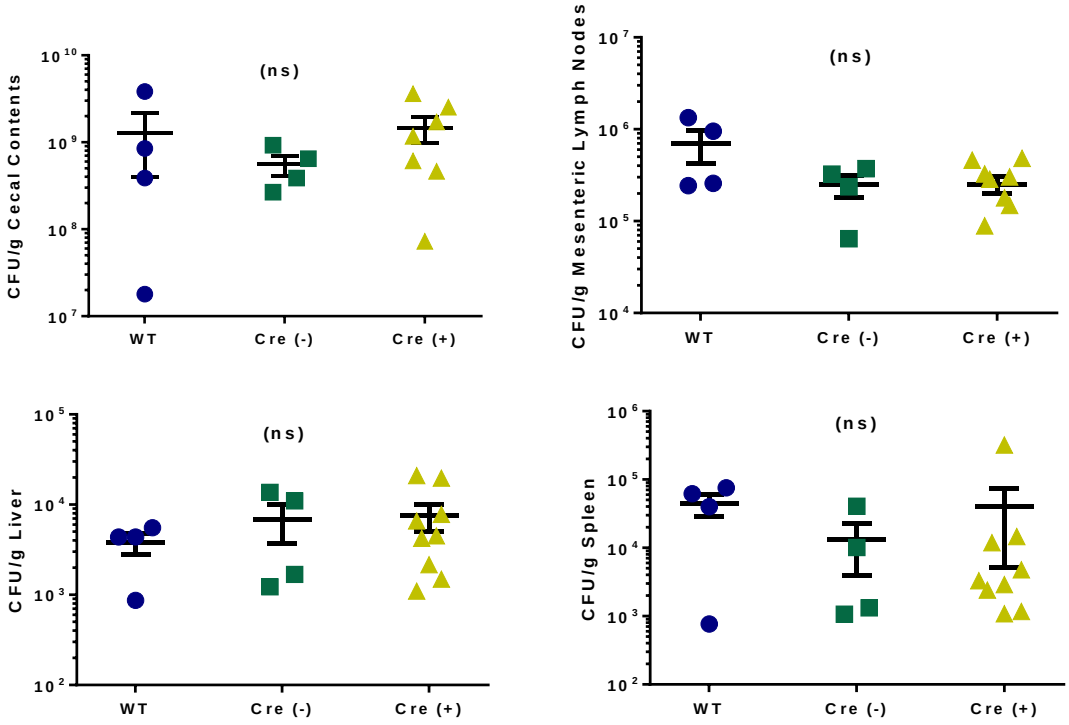

Figure 19. Mouse Experiment 2: Bacterial Load in Mouse Cecum Contents and Tissues 48 Hours Post-Infection with *S.* Typhimurium. Symbols represent individual mice. Analyzed with one-way ANOVA and Tukey's multiple comparisons test.

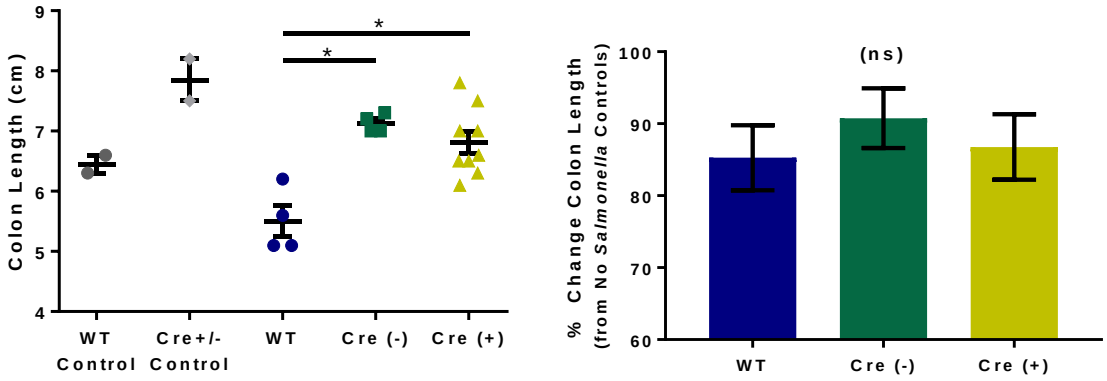

Figure 20. Mouse Experiment 2: Colon Length 48 Hours Post-Infection with *S.* Typhimurium. Symbols represent individual mice. (A) Analyzed with one-way ANOVA and Tukey's multiple comparisons test. * denotes $p<0.05$. (B) Normalized colon lengths of *Salmonella*-exposed mice to the streptomycin-gavaged, colony-matched control mouse colon lengths. Analyzed with one-way ANOVA

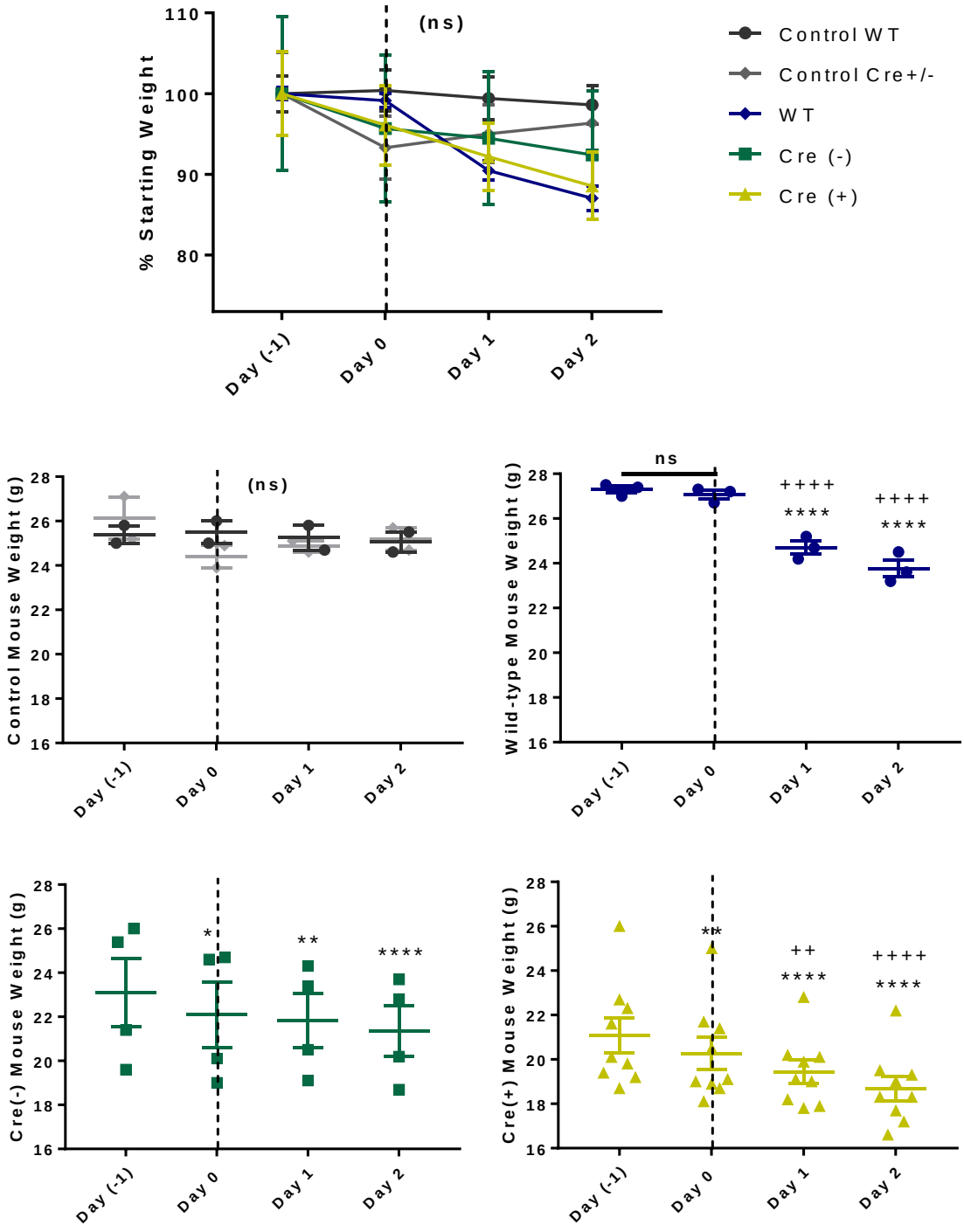

Figure 21. Mouse Experiment 2: Mouse Weight Over Time and Colon Length 48 Hours Post-Infection with *S.* Typhimurium. Analyzed via two-way ANOVA with Tukey's multiple comparisons tests. * denotes difference from day -1 (before streptomycin gavage), + denotes difference from day 0 (before *Salmonella* gavage).

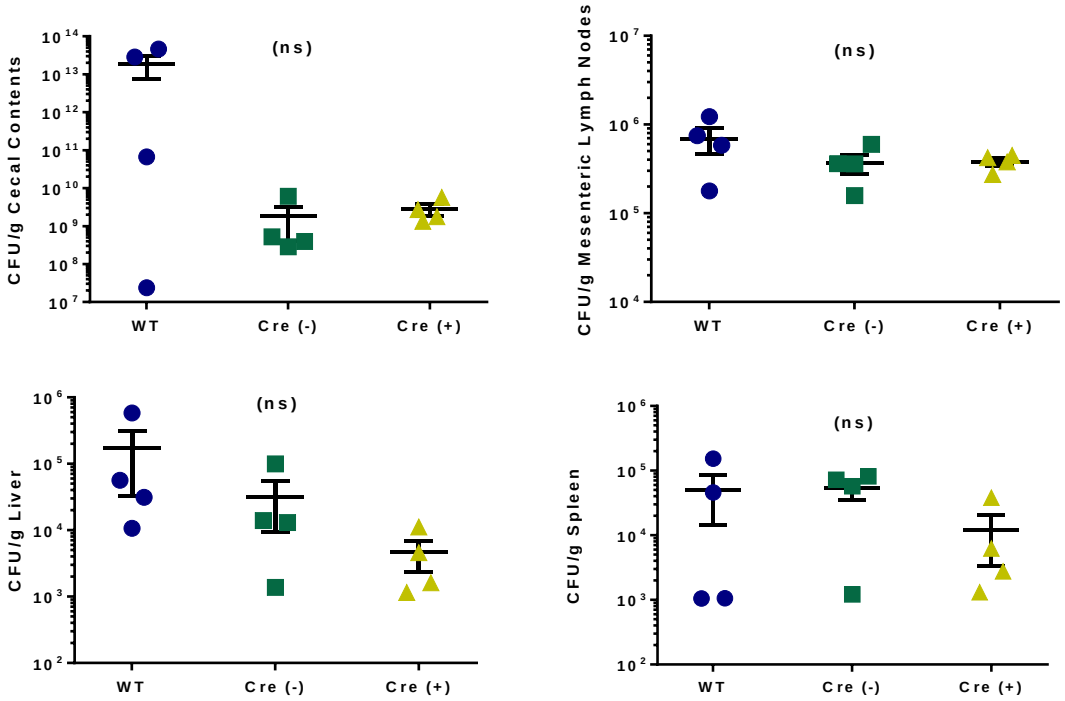

Figure 22. Mouse Experiment 3: Bacterial Load in Mouse Cecum Contents and Tissues 48 Hours Post-Infection with *S.* Typhimurium mCherry. Symbols represent individual mice. Analyzed with one-way ANOVA and Tukey's multiple comparisons test.

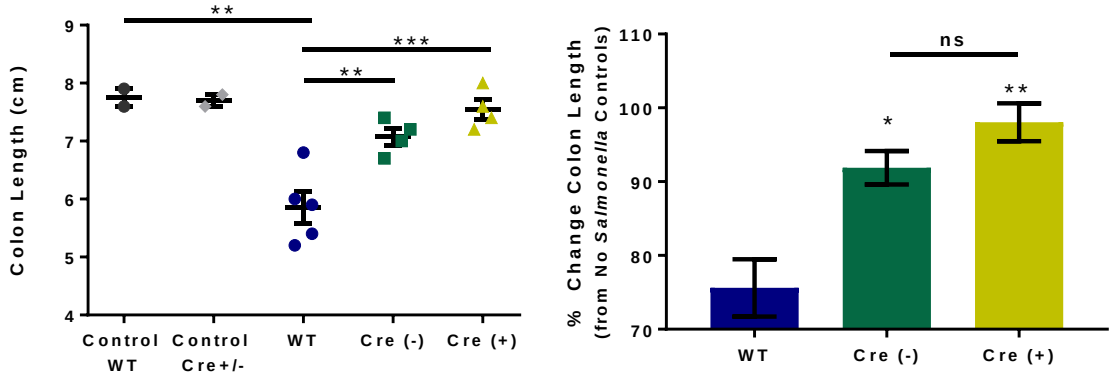

Figure 23. Mouse Experiment 3: Colon Length 48 Hours Post-Infection with *S.* Typhimurium mCherry. Symbols represent individual mice. (A) **, *** denotes $p<0.01$, $p<0.001$ between indicated groups. (B) *, ** denotes $p<0.05$, $p<0.01$ from wild-type. Analyzed with one-way ANOVA and Tukey's multiple comparisons test.

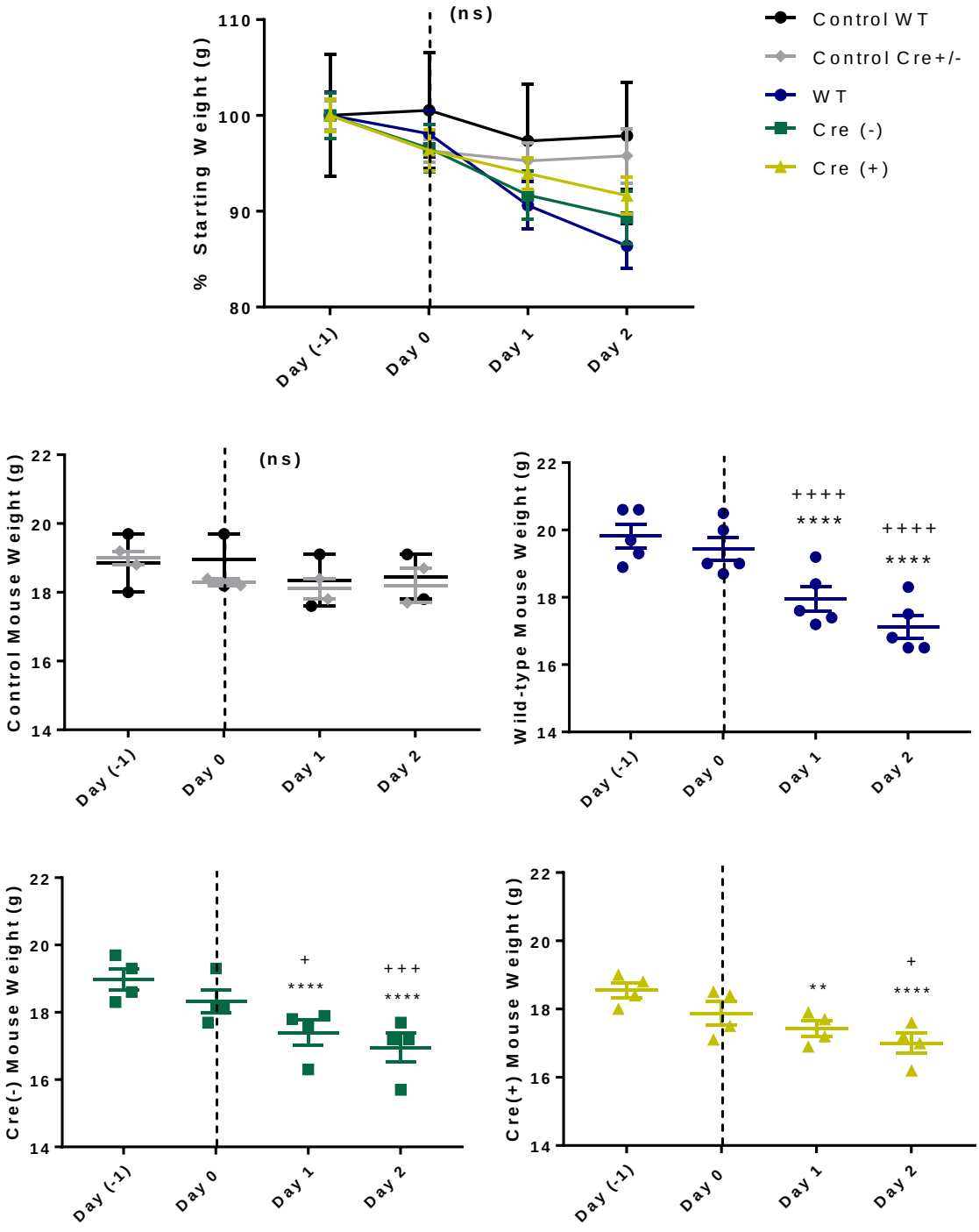

Figure 24. Mouse Experiment 3: Mouse Weight Over Time Through 48 Hour Infection with *S.* Typhimurium mCherry. Analyzed via two-way ANOVA with Tukey's multiple comparisons tests. * denotes difference from day -1 (before streptomycin gavage), + denotes difference from day 0 (before *Salmonella* gavage).

Table 6. Species Identification and Antimicrobial Susceptibility Results for Unknown Streptomycin-Resistant Bacterium Recovered from $CD73^{f/f}Villin^{Cre}$ Mice. (Top) Species identification of 2 unknown streptomycin-resistant bacterial colonies from $CD73^{f/f}Villin^{Cre}$ mice from Mouse Experiment 3. Antimicrobial susceptibility testing was performed on Isolate #1. (Bottom) Species identification and antimicrobial susceptibility panels of 3 unknown streptomycin-resistant bacterial colonies from streptomycin-gavaged $CD73^{f/f}Villin^{Cre}$ mice.

Aerobic Culture

Animal ID	Specimen	Isolate #	Organism	Amount
	agar plate	1	Enterococcus faecalis	4+
	agar plate		Enterococcus faecalis	4+

ANTIMICROBIAL SUSCEPTIBILITY

Drug	*(enfaecal)* 1
Amoxicillin-clavulanate	S
Ampicillin	S
Chloramphenicol	I
Erythromycin	I
Nitrofurantoin	S
Penicillin	S
Tetracycline	S

S = Sensitive, R = Resistant, I = Intermediate

Aerobic Culture

Animal ID	Specimen	Isolate #	Organism	Amount
141	agar plate	1	Enterococcus faecalis	2+
142	agar plate	2	Enterococcus faecalis	4+
143	agar plate	3	Enterococcus faecalis	4+

ANTIMICROBIAL SUSCEPTIBILITY

Drug	*(enfaecal)* 1	*(enfaecal)* 2	*(enfaecal)* 3
Amoxicillin-clavulanate	S	S	S
Ampicillin	S	S	S
Chloramphenicol	I	I	I
Erythromycin	R	I	R
Nitrofurantoin	I	S	S
Penicillin	S	S	S
Tetracycline	I	I	I

S = Sensitive, R = Resistant, I = Intermediate

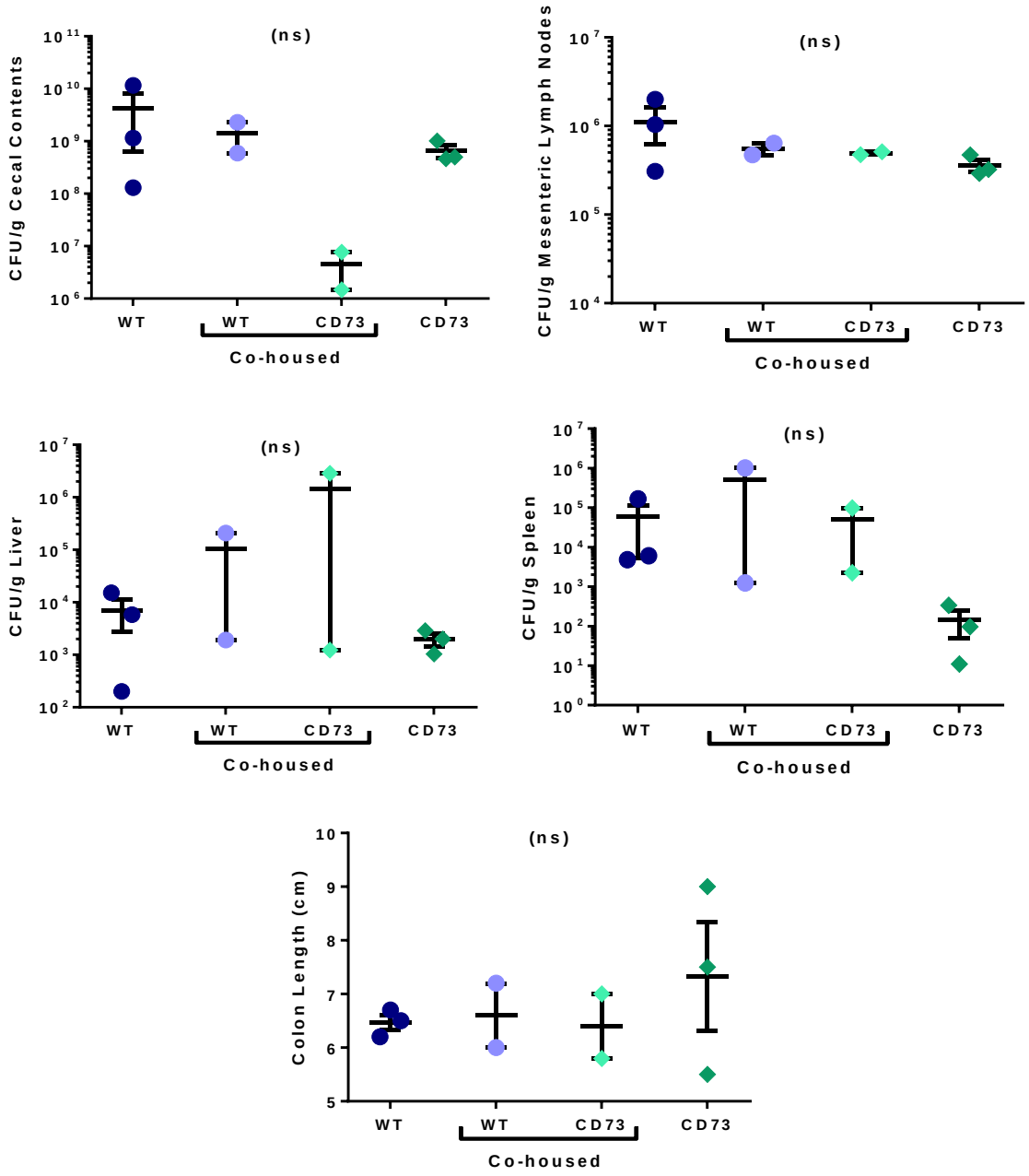

Figure 25. Mouse Experiment 4: Co-housed Mouse Colon Length and Bacterial Load in Cecum Contents and Tissues 48 Hours Post-Infection with *S.* Typhimurium mCherry. Symbols represent individual mice. Analyzed with one-way ANOVA and Tukey's multiple comparisons test.

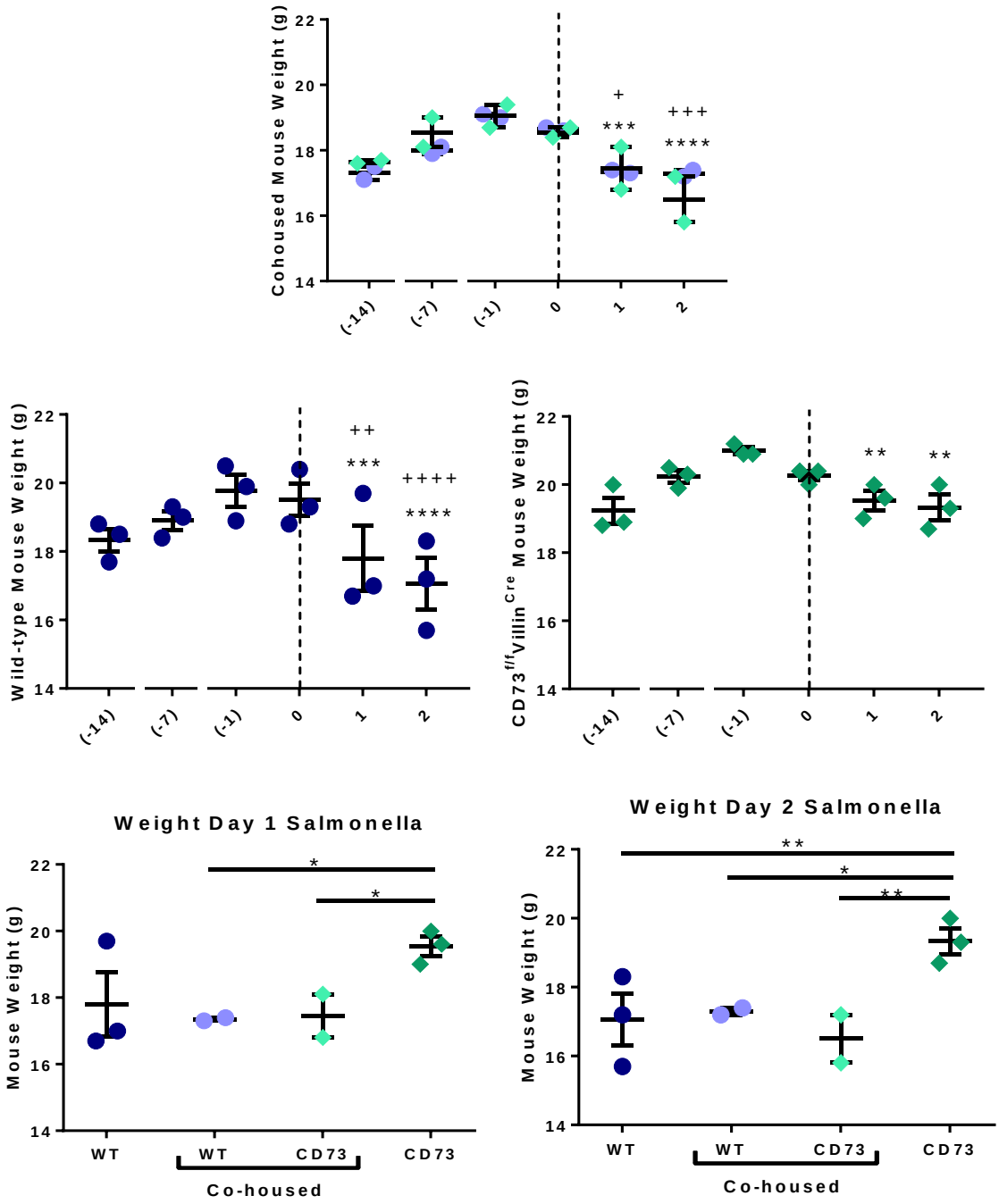

Figure 26. Mouse Experiment 4: Co-housed Mouse Weight Over Time Through 48 Hour Infection with *S.* Typhimurium mCherry. Analyzed via two-way ANOVA with Tukey's multiple comparisons tests. (A-C) * denotes difference from day -1 (before streptomycin gavage), + denotes difference from day 0 (before *Salmonella* gavage). (D,E) Results plotted by individual days. *, ** indicates $p<0.05$, $p<0.01$.

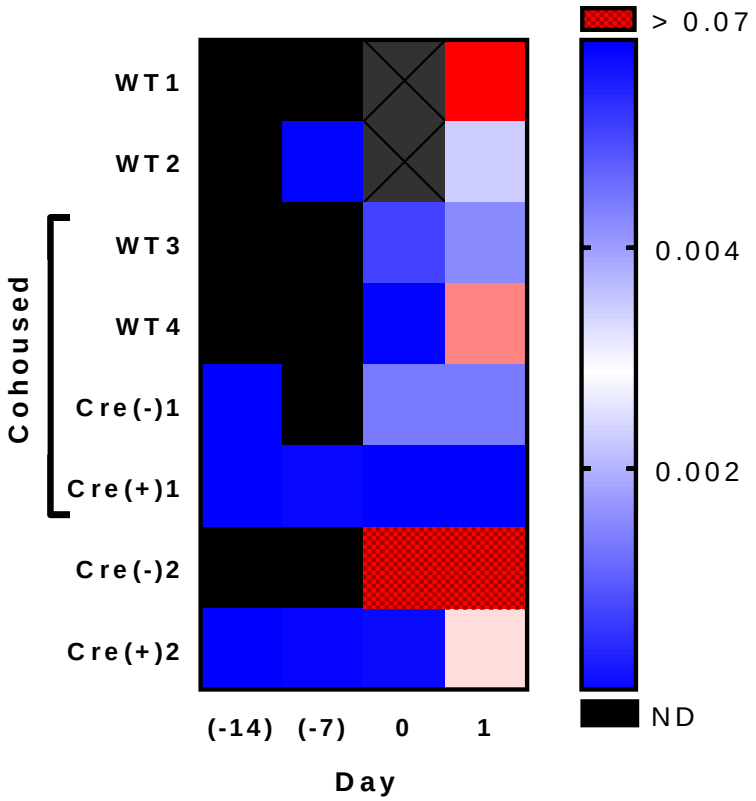

Figure 27. Heat Map of *E. faecalis* Relative Abundance in Singly-housed and Cohoused Mouse Fecal Samples. *E. faecalis* 16S levels were normalized to total bacterial 16S recovered from mouse fecal samples. Day (-14) samples were taken before cohousing, Day (-7) samples were taken after one week of cohousing, Day 0 samples were taken 24 hours-post streptomycin gavage, and Day 1 samples were taken 24 hours-post *S.* Typhimurium SL1344 mCherry gavage. Black boxes indicate *E. faecalis* was not detectable in the sample. Crossed out boxes for WT1 and WT2 on Day 0 indicate DNA was unable to be recovered fecal samples.

Conclusions

The studies outlined in this thesis were unable to reliably recapitulate the findings by Kao and colleagues that demonstrated a role for IEC-specific CD73 in modulating *S.* Typhimurium virulence [3]. However, it is quite possible that the lack of consistent experimental reproducibility was due to confounding factors that appeared in both *in vitro* and *in vivo* models.

Using the gentamicin protection assay to assess IEC invasion and intracellular replication by *S.* Typhimurium, only twice was enough *Salmonella* recovered from control T84 cells to meet the criteria for a successful assay (Figure 1, Figure 3). Since *S.* Typhimurium could not reliably replicate in control cells, it was not possible to properly assess the role of CD73 in modulating *S.* Typhimurium virulence in the T84 cell culture model. However, there was a difference observed in IL-8 production from shCD73 T84 cells compared to controls after 10 h.p.i. with *S.* Typhimurium (Figure 13) and there was never any bacterial replication measured in shCD73 T84 cells, so these studies do provide some evidence to suggest a role for CD73-dependent host responses to *S.* Typhimurium in the epithelium.

While T84 cells may not stand as a robust model for assessing the role of CD73 in *S.* Typhimurium infection, there are at least two other cell culture models in our lab that may be good candidates for the development of new *in vitro* models to test the function of CD73 in human IECs. It was demonstrated that C2BBe1 cells can support robust growth of *S.* Typhimurium (Figure 14) but since they express relatively low levels of CD73 at baseline, a CD73 overexpression cell line would need to be created in these cells

(Figure 15). Also, although the ability for HT29 cells to support *S.* Typhimurium replication was not verified here, they are a widely-used model for studying *S.* Typhimurium invasion and replication and they express comparable levels of CD73 to T84 cells (Figure 15) [161].

Difficulties encountered in the *in vivo* experiments utilizing the streptomycin-pretreatment model of *Salmonella* colitis were multifaceted and, again, prevented these studies from fully recapitulating the phenotype reported by Kao and colleagues. No significant differences in luminal or tissue *Salmonella* load were discovered between any groups treated with *S.* Typhimurium in any of the mouse infection experiments. There were also no differences discovered between the colon lengths or weight of Salmonella-exposed Cre (-) and Cre (+) mice in any experiment performed in this facility. However, there were two identified factors that introduced potentially confounding variables into the streptomycin-pretreatment model that may have masked the influence of CD73 on *S.* Typhimurium virulence in the intestinal epithelium.

First, it was discovered that some mice in the $CD73^{f/f}$ $Villin^{Cre}$ mouse colony were total body knockout for CD73, probably due to leaky Cre recombinase expression in the testes of Cre (+) male mice used in breeding pairs (H. Grifka-Walk, unpublished data) [128]. Therefore, animal husbandry practices were altered so that only $CD73^{f/f}$ $Villin^{Cre-}$ males and $CD73^{f/f}$ $Villin^{Cre+}$ females were used for breeding pairs to generate Cre (+) and Cre (-) mice. The genotyping protocol was also updated to include an assay to detect mice harboring a null allele of CD73 (Table 2). Once the issues without mouse genetics were addressed, *E. faecalis* was discovered in the intestinal microbiomes of the mouse

colony. *E. faecalis* is a common member of the mouse microbiome, but these experiments suggest that *E. faecalis* may be present in higher numbers in the microbiomes of the $CD73^{f/f}$ $Villin^{Cre}$ mice compared to wild-type mice. More importantly, other researchers have shown that *E. faecalis* can provide protection against *Salmonella* infection, so that may explain why there were no genotype-specific effects uncovered.

In summary, these studies were unable to validate the results reported by Kao et al. demonstrating an IEC-specific role of CD73 in *S.* Typhimurium virulence modulation, but this was most likely due to difficulties encountered in establishing the models used by these authors in our facility [3]. Further work on this project will likely rely upon the establishment of new *in vitro* and *in vivo* models to define a role for IEC-specific CD73 in *S.* Typhimurium virulence modulation.

FUTURE DIRECTIONS

Future work on this project could contribute significantly to our understanding of the mechanism by which CD73 modulates *S.* Typhimurium pathogenesis, potentially revealing candidate targets for therapeutic modulation of invasive inflammatory pathogens in the gut mucosa. Although much of the data presented in this thesis was inconclusive, it does demonstrate that new methods may need to be used in order to extend the findings presented by Kao and colleagues [3]. Fortunately, there are several promising alternative *in vitro* and *in vivo* models that could easily be adopted by our lab to rejuvenate this project investigating the role of CD73 in the intestinal epithelium.

Developing Alternate Human Intestinal Epithelial Cell Culture Models to Study the Role of CD73 in *S.* Typhimurium Virulence

Alternate *In Vitro* Models

T84 cells were chosen as our first *in vitro* model system because this was the original human enterocyte cell line used in characterizing the apical, robust expression of CD73 in IECs, plus this cell line was occasionally used to study *S.* Typhimurium pathogenesis [3], [81], [130]. However, as my data have shown, the T84 cells in our laboratory may not support robust replication of *S.* Typhimurium at early times post infection compared to other immortalized cell types used to study invasion and replication in IECs. Additionally, since there are few published experiments that characterize *S.* Typhimurium infection in T84 cells, adopting a more widely-used *in vitro* model may help us to better compare our findings and overcome some of the obstacles encountered in using these T84s.

Caco-2, C2BBe1, and HT-29 are all commonly used cell lines in studies investigating *Salmonella* infection in human IECs. As discussed above, since Caco-2 and C2BBe1 cells express a low amount of CD73 at baseline, overexpressing cell lines would need to be developed in order to investigate the role of CD73 in disease pathogenesis (Figure 15). However, I suspect that *Salmonella* invasion and intracellular replication would exert more subtle differences in an overexpressing cell line compared to the knockdown phenotype. I hypothesize that an increase of CD73 expression would provide only a modest benefit to *Salmonella* replication because of a saturation point for CD73-generated adenosine, which may make CD73-dependent differences more difficult to measure than in the shCD73 knockdown cells. Fortunately, I measured a high level of CD73 expression in HT29 cells, which makes them a promising candidate for recreating an shCD73 knockdown cell line with which to study *S.* Typhimurium virulence modulation by CD73 in human IECs.

Defining Mechanisms of Virulence Modulation

Once a suitable *in vitro* model is developed, it can be used to address a multitude of questions that remain on how CD73 modulates *S.* Typhimurium infection in IECs. In order to begin to discern the mechanism(s) of *S.* Typhimurium virulence modulation by CD73, it would be helpful to understand whether the lack of CD73 in IECs is primarily affecting change in host gene regulation or *Salmonella* gene regulation. Differences in host versus *Salmonella* effects can start to be elucidated by utilizing the gentamicin protection assay in combination with fluorescence microscopy and RT-qPCR experiments.

Kao et al. have shown that in the absence of CD73, *S.* Typhimurium becomes localized to the apical side of polarized human colonic epithelial cells [3]. This finding taken with previous observations noting decreased bacterial replication and translocation in the absence of CD73 suggest *S.* Typhimurium effectors are impaired in their ability to coordinate early infection events, such as stabilization of or escape from an SCV. It is currently unknown whether *S.* Typhimurium invades shCD73 IECs in a typical SPI-1-dependent process or whether they invade via a SPI-1-independent mechanism that is affecting their ability to replicate intracellularly. Since the host and *Salmonella* genes necessary for establishing and maintaining an SCV have been well-characterized using fluorescence microscopy, we could look at shCD73 and control IEC monolayers to determine any overt differences in *S.* Typhimurium invasion, localization, and SCV formation. Additionally, our collaborators at Washington State University are experts in confocal microscopy and possess unique *S.* Typhimurium isolates and a wide array of microscopy reagents and techniques that allow them to determine subtle differences in *S.* Typhimurium invasion, translocation, and replication in IECs [99].

Next, it is unknown whether a CD73 inhibitor recapitulates the *Salmonella* virulence attenuation phenotype observed in shCD73 cells. If so, this would indicate that acute adenosine concentrations are important for the CD73-linked *S.* Typhimurium virulence modulation. If not, it would suggest some long-term adaptive response to compensate for the lack of CD73 may be responsible for inhibiting intracellular *Salmonella* replication. To test this, a gentamicin protection could be carried out in shCD73 and control IECs with and without the CD73 inhibitor α,β-methylene ADP to

see if the bacteria recovered from control cells treated with the inhibitor is comparable to the amount of *Salmonella* recovered from shCD73 cells over time. Metabolomic analyses of adenosine along with upstream and downstream adenosine metabolites could be used to support and extend findings uncovered by these experiments.

It is also unknown if and how shCD73 IECs alter their adenosine-related regulatory pathways at baseline and upon exposure to *Salmonella.* An RT-qPCR screen could be performed on *Salmonella*-infected shCD3 and control IEC monolayers to examine the expression of host genes likely to be altered by decreased extracellular adenosine generation before and throughout infection. The human RT-qPCR primer sets for those genes involved in alternate pathways for adenosine generation, transport, and signaling, including cytosolic 5'-nucleotidase (NT5C), intestinal alkaline phosphatase (IAP), adenosine kinase (ADK), equilibrative nucleoside transporters (ENTs), concentrative nucleoside transporters (CNTs), and adenosine receptors (ADORAs), are easily available online for our use. We could also follow up these results by measuring protein expression, performing enzyme activity assays, or using different inhibitors throughout a gentamicin protection assay to confirm candidate genes.

Finally, we could examine the expression of *S.* Typhimurium genes that are important for coordinating early infection events before and throughout a gentamicin protection assay using shCD73 and control IEC monolayers. SPI-1 effectors of interest include SipA, SopA, SopB, SopD, SopE and SopE2 sine they are important for IEC invasion events [71], [162]. SipA coordinates the timing of IEC invasion, Sop B, SopE, and SopE2 work to modulate host RhoGTPase functions to direct actin cytoskeleton

rearrangements, and SopD promotes macropinosome closure [162], [163]. SPI-2 effectors of interest include: SpiC, which interferes with vesicular trafficking, SifA, which is required for SCV membrane integrity and Sif formation, SopD2, which inhibits the GTPase Rab7 from directing endocytosis of vacuoles, and SseJ, which modifies proteins on the SCV membrane to allow for proper replication in SCVs [100], [164]–[166].

The experiments proposed here will contribute significantly to our understanding of host-pathogen interactions involving *S.* Typhimurium and IEC-specific CD73. Furthermore, our lab and our collaborators have extensive experience with the proposed techniques so these experiments are feasible upon the development of a new *in vitro* model to study the role of CD73 in IECs.

Establishing a Model of *Salmonella* Colitis at Montana State University

Although it is a well-used model to study *S.* Typhimurium, my data suggest the streptomycin-pretreatment model of *Salmonella* colitis may not be a suitable model system to study *S.* Typhimurium infection in mice from our facility at Montana State University. It is well known that the composition of intestinal microbiome is key to the success of this model and several attempts have been made to understand the specific factors leading to the shift in *S.* Typhimurium disease presentation in mice with and without streptomycin pretreatment [58], [167], [168]. However, we simply need an alternate model of *Salmonella* infection with which to test the mechanism of CD73 virulence modulation in the intestinal epithelium. Fortunately, several other models have been described that could easily be adapted to our facility.

One approach may be to change how the antibiotics are administered to mice. Currently, with the streptomycin-pretreatment model, mice receive a single, high-dose (20 mg) dose of streptomycin administered via oral gavage that severely disrupts the microbial ecosystem of the gut microbiota [58], [168]. Sekirov and colleagues have developed a low-dose streptomycin treatment model, in which mice are administered a lower dose of streptomycin in their drinking water two days prior to *Salmonella* in order to decrease the microbial community disruption caused by high-dose streptomycin administration. In this model, low-dose streptomycin water contains 460mg/L streptomycin with mice receiving an estimated average dose of roughly 1.35mg streptomycin per day and the authors were able to validate this model by detecting subtle differences in *Salmonella* invasion mutants [84]. Perhaps the low-dose model could be used in our studies to decrease the intestinal perturbation caused by a single, high-dose of streptomycin which could mitigate *E. faecalis* overgrowth after streptomycin-pretreatment. However, low-dose administration still may select for streptomycin-resistant *E. faecalis* which we believe is disruptive to our current model, so our lab may have to move away from utilizing streptomycin in our mice as a means of overcoming colonization resistance for *S.* Typhimurium.

There are two promising options immediately available to us for use in our facility that could be used to establish a different model of *Salmonella* colitis in the absence of streptomycin: use a different antibiotic to disrupt the intestinal microbial ecosystem or use gnotobiotic mice. The easiest approach of these options is simply changing out the antibiotic used to induce intestinal dysbiosis in mice. The same authors

that described the low-dose streptomycin model also described the effects of low-dose vancomycin administration to mice in their drinking water before infection with *S.* Typhimurium [168]. However, we would want to first test the vancomycin susceptibility of *E. faecalis* before initiating these experiments to ensure we would not yet again be inducing *E. faecalis* overgrowth. The use of germ-free mice as a *Salmonella* infection model has also been described and using this model would eliminate the need for troubleshooting microbiome- and antibiotic-related effects of *Salmonella* infection [167]. Since we have access to a germ-free mouse facility and the ability to derive mouse lines germ-free, the gnotobiotic model of *Salmonella* infection may be worthy of consideration. One potential pitfall to the gnotobiotic *Salmonella* infection model for our purposes is that we are unaware of the feasibility of deriving $CD73^{f/f}$ $Villin^{Cre}$ mice germ-free, since this has yet to be reported.

In summary, the original streptomycin-pretreatment model of *Salmonella* colitis imparts an advantage to other murine models due to the wide-spread used of this model, but it is becoming clear that future work in our facility may necessitate revisiting these other models.

REFERENCES CITED

[1] M. S. Bynoe, A. T. Waickman, D. A. Mahamed, C. Mueller, J. H. Mills, and A. Czopik, “CD73 Is Critical for the Resolution of Murine Colonic Inflammation,” *J. Biomed. Biotechnol.*, vol. 2012, 2012.

[2] M. S. Alam *et al.*, “Ecto-5′-Nucleotidase (CD73) Regulates Host Inflammatory Responses and Exacerbates Murine Salmonellosis,” *Sci. Rep.*, vol. 4, Mar. 2014.

[3] D. J. Kao *et al.*, “Intestinal epithelial ecto-5’-nucleotidase (CD73) regulates intestinal colonization and infection by nontyphoidal Salmonella,” *Infect. Immun.*, In Press 2017.

[4] P. Koszalka *et al.*, “Targeted Disruption of cd73/Ecto-5′-Nucleotidase Alters Thromboregulation and Augments Vascular Inflammatory Response,” *Circ. Res.*, vol. 95, no. 8, pp. 814–821, Oct. 2004.

[5] G. Haskó and B. N. Cronstein, “Adenosine: an endogenous regulator of innate immunity,” *Trends Immunol.*, vol. 25, no. 1, pp. 33–39, Jan. 2004.

[6] S. M. Dann and L. Eckmann, “Innate immune defenses in the intestinal tract,” *Curr. Opin. Gastroenterol.*, vol. 23, no. 2, pp. 115–120, Mar. 2007.

[7] A. M. O’Hara and F. Shanahan, “The gut flora as a forgotten organ,” *EMBO Rep.*, vol. 7, no. 7, pp. 688–693, Jul. 2006.

[8] E. P. DiMagno, V. L. W. Go, and W. H. J. Summerskill, “Relations between Pancreatic Enzyme Outputs and Malabsorption in Severe Pancreatic Insufficiency,” *N. Engl. J. Med.*, vol. 288, no. 16, pp. 813–815, Apr. 1973.

[9] P. G. Lankisch, B. Lembcke, G. Wemken, and W. Creutzfeldt, “Functional Reserve Capacity of the Exocrine Pancreas,” *Digestion*, vol. 35, no. 3, pp. 175–181, 1986.

[10] A. F. Hofmann, “The Continuing Importance of Bile Acids in Liver and Intestinal Disease,” *Arch. Intern. Med.*, vol. 159, no. 22, pp. 2647–2658, Dec. 1999.

[11] A. F. Hofmann and L. Eckmann, “How bile acids confer gut mucosal protection against bacteria,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 12, pp. 4333–4334, Mar. 2006.

[12] F. Raul, F. Gosse, M. Doffoel, P. Darmenton, and J. Y. Wessely, “Age related increase of brush border enzyme activities along the small intestine.,” *Gut*, vol. 29, no. 11, pp. 1557–1563, Nov. 1988.

[13] B. E. Goodman, “Insights into digestion and absorption of major nutrients in humans,” *Adv. Physiol. Educ.*, vol. 34, no. 2, pp. 44–53, Jun. 2010.

[14] H. F. Helander and L. Fändriks, “Surface area of the digestive tract – revisited,” *Scand. J. Gastroenterol.*, vol. 49, no. 6, pp. 681–689, Jun. 2014.

[15] R. Randal Bollinger, A. S. Barbas, E. L. Bush, S. S. Lin, and W. Parker, “Biofilms in the large bowel suggest an apparent function of the human vermiform appendix,” *J. Theor. Biol.*, vol. 249, no. 4, pp. 826–831, Dec. 2007.

[16] J. J. Galligan, “Fecal propulsion in the colon: what a long, strange trip it is!,” *J. Physiol.*, vol. 586, no. Pt 17, p. 4029, Sep. 2008.

[17] J. L. Round and S. K. Mazmanian, “The gut microbiome shapes intestinal immune responses during health and disease,” *Nat. Rev. Immunol.*, vol. 9, no. 5, pp. 313–323, May 2009.

[18] J. R. Turner, “Intestinal mucosal barrier function in health and disease,” *Nat. Rev. Immunol.*, vol. 9, no. 11, pp. 799–809, Nov. 2009.

[19] G. E. Sandusky, S. F. Badylak, R. J. Morff, W. D. Johnson, and G. Lantz, “Histologic findings after in vivo placement of small intestine submucosal vascular grafts and saphenous vein grafts in the carotid artery in dogs.,” *Am. J. Pathol.*, vol. 140, no. 2, pp. 317–324, Feb. 1992.

[20] D. King, “Southern Illinois University School of Medicine Histology of the Gastrointestinal System,” 2009. [Online]. Available: http://www.siumed.edu/~dking2/erg/giguide.htm. [Accessed: 08-Jul-2017].

[21] H. Nakajima-Adachi *et al.*, “Peyer’s Patches and Mesenteric Lymph Nodes Cooperatively Promote Enteropathy in a Mouse Model of Food Allergy,” *PLOS ONE*, vol. 9, no. 10, p. e107492, Oct. 2014.

[22] G. Eberl and M. Lochner, “The development of intestinal lymphoid tissues at the interface of self and microbiota,” *Mucosal Immunol.*, vol. 2, no. 6, pp. 478–485, Sep. 2009.

[23] I. Spadoni, A. Pietrelli, G. Pesole, and M. Rescigno, “Gene expression profile of endothelial cells during perturbation of the gut vascular barrier,” *Gut Microbes*, vol. 7, no. 6, pp. 540–548, Nov. 2016.

[24] J. B. Furness, *The Enteric Nervous System*. Victoria, Australia: Blackwell Publishing, 2006.

[25] M. F. Cesta, “Normal Structure, Function, and Histology of Mucosa-Associated Lymphoid Tissue,” *Toxicol. Pathol.*, vol. 34, no. 5, pp. 599–608, Aug. 2006.

[26] N. Takemura *et al.*, "Blockade of TLR3 protects mice from lethal radiation-induced gastrointestinal syndrome," *Nat. Commun.*, vol. 5, p. ncomms4492, Mar. 2014.

[27] K. R. Groschwitz and S. P. Hogan, "Intestinal Barrier Function: Molecular Regulation and Disease Pathogenesis," *J. Allergy Clin. Immunol.*, vol. 124, no. 1, pp. 3–22, Jul. 2009.

[28] N. Khan and A. R. Asif, "Transcriptional Regulators of Claudins in Epithelial Tight Junctions," *Mediators Inflamm.*, vol. 2015, 2015.

[29] A. M. Costa, M. Leite, R. Seruca, and C. Figueiredo, "Adherens junctions as targets of microorganisms: A focus on Helicobacter pylori," *FEBS Lett.*, vol. 587, no. 3, pp. 259–265, Jan. 2013.

[30] H. K. Campbell, J. L. Maiers, and K. A. DeMali, "Interplay between tight junctions & adherens junctions," *Exp. Cell Res.*, vol. 358, no. 1, pp. 39–44, Sep. 2017.

[31] O. Nekrasova and K. J. Green, "Desmosome assembly and dynamics," *Trends Cell Biol.*, vol. 23, no. 11, pp. 537–546, Nov. 2013.

[32] P. D. Lampe and A. F. Lau, "The effects of connexin phosphorylation on gap junctional communication," *Int. J. Biochem. Cell Biol.*, vol. 36, no. 7, pp. 1171–1186, Jul. 2004.

[33] L. A. Chia and C. J. Kuo, "The Intestinal Stem Cell," *Prog. Mol. Biol. Transl. Sci.*, vol. 96, pp. 157–173, 2010.

[34] C. Metcalfe, N. M. Kljavin, R. Ybarra, and F. J. de Sauvage, "Lgr5+ Stem Cells Are Indispensable for Radiation-Induced Intestinal Regeneration," *Cell Stem Cell*, vol. 14, no. 2, pp. 149–159, Feb. 2014.

[35] N. Barker *et al.*, "Identification of stem cells in small intestine and colon by marker gene Lgr5," *Nature*, vol. 449, no. 7165, pp. 1003–1007, Oct. 2007.

[36] Y. Sasai, M. Eiraku, and H. Suga, "In vitro organogenesis in three dimensions: self-organising stem cells," *Development*, vol. 139, no. 22, pp. 4111–4121, Nov. 2012.

[37] N. Barker, A. van Oudenaarden, and H. Clevers, "Identifying the Stem Cell of the Intestinal Crypt: Strategies and Pitfalls," *Cell Stem Cell*, vol. 11, no. 4, pp. 452–460, Oct. 2012.

[38] F. Gerbe, C. Legraverend, and P. Jay, “The intestinal epithelium tuft cells: specification and function,” *Cell. Mol. Life Sci.*, vol. 69, no. 17, pp. 2907–2917, Sep. 2012.

[39] P. De Santa Barbara, G. R. Van Den Brink, and D. J. Roberts, “Development and differentiation of the intestinal epithelium,” *Cell. Mol. Life Sci.*, vol. 60, no. 7, pp. 1322–1332, Jul. 2003.

[40] T. Pelaseyed *et al.*, “The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system,” *Immunol. Rev.*, vol. 260, no. 1, pp. 8–20, Jul. 2014.

[41] N. Miron and V. Cristea, “Enterocytes: active cells in tolerance to food and microbial antigens in the gut,” *Clin. Exp. Immunol.*, vol. 167, no. 3, pp. 405–412, Mar. 2012.

[42] B. Corthesy, “Multi-Faceted Functions of Secretory IgA at Mucosal Surfaces,” *Front. Immunol.*, vol. 4, 2013.

[43] V. Snoeck, B. Goddeeris, and E. Cox, “The role of enterocytes in the intestinal barrier function and antigen uptake,” *Microbes Infect.*, vol. 7, no. 7, pp. 997–1004, Jun. 2005.

[44] W. O. Dobbins, “Human intestinal intraepithelial lymphocytes.,” *Gut*, vol. 27, no. 8, pp. 972–985, Aug. 1986.

[45] B. S. Sheridan and L. Lefrançois, “Intraepithelial Lymphocytes: To Serve and Protect,” *Curr. Gastroenterol. Rep.*, vol. 12, no. 6, pp. 513–521, Dec. 2010.

[46] N. A. Mabbott, D. S. Donaldson, H. Ohno, I. R. Williams, and A. Mahajan, “Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium,” *Mucosal Immunol.*, vol. 6, no. 4, pp. 666–677, Jul. 2013.

[47] T. Kucharzik *et al.*, “Role of M cells in intestinal barrier function,” *Ann. N. Y. Acad. Sci.*, vol. 915, pp. 171–183, 2000.

[48] B. D. Jones, N. Ghori, and S. Falkow, “Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer9s patches.,” *J. Exp. Med.*, vol. 180, no. 1, pp. 15–23, Jul. 1994.

[49] R. D. Specian and M. G. Oliver, “Functional biology of intestinal goblet cells,” *Am. J. Physiol. - Cell Physiol.*, vol. 260, no. 2, pp. C183–C193, Feb. 1991.

[50] J. B. Furness, W. A. A. Kunze, and N. Clerc, “II. The intestine as a sensory organ: neural, endocrine, and immune responses,” *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 277, no. 5, pp. G922–G928, Nov. 1999.

[51] C. Sternini, L. Anselmi, and E. Rozengurt, “Enteroendocrine cells: a site of ‘taste’ in gastrointestinal chemosensing,” *Curr. Opin. Endocrinol. Diabetes Obes.*, vol. 15, no. 1, pp. 73–78, Feb. 2008.

[52] M. R. Howitt *et al.*, “Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut,” *Science*, vol. 351, no. 6279, pp. 1329–1333, Mar. 2016.

[53] R. K. Grencis and J. J. Worthington, “Tuft Cells: A New Flavor in Innate Epithelial Immunity,” *Trends Parasitol.*, vol. 32, no. 8, pp. 583–585, Aug. 2016.

[54] I. I. Ivanov *et al.*, “Specific microbiota direct the differentiation of Th17 cells in the mucosa of the small intestine,” *Cell Host Microbe*, vol. 4, no. 4, pp. 337–349, Oct. 2008.

[55] J. R. McGhee and K. Fujihashi, “Inside the Mucosal Immune System,” *PLOS Biol.*, vol. 10, no. 9, p. e1001397, Sep. 2012.

[56] T. D. Lawley and A. W. Walker, “Intestinal colonization resistance,” *Immunology*, vol. 138, no. 1, pp. 1–11, Jan. 2013.

[57] D. Ribet and P. Cossart, “How bacterial pathogens colonize their hosts and invade deeper tissues,” *Microbes Infect.*, vol. 17, no. 3, pp. 173–183, Mar. 2015.

[58] C. D. Garner *et al.*, “Perturbation of the Small Intestine Microbial Ecology by Streptomycin Alters Pathology in a Salmonella enterica Serovar Typhimurium Murine Model of Infection,” *Infect. Immun.*, vol. 77, no. 7, pp. 2691–2702, Jul. 2009.

[59] M. Barthel *et al.*, “Pretreatment of Mice with Streptomycin Provides a Salmonella enterica Serovar Typhimurium Colitis Model That Allows Analysis of Both Pathogen and Host,” *Infect. Immun.*, vol. 71, no. 5, pp. 2839–2858, May 2003.

[60] E. V. Gart, J. S. Suchodolski, T. H. Welsh, R. C. Alaniz, R. D. Randel, and S. D. Lawhon, “Salmonella Typhimurium and Multidirectional Communication in the Gut,” *Front. Microbiol.*, vol. 7, Nov. 2016.

[61] M. Barman *et al.*, “Enteric Salmonellosis Disrupts the Microbial Ecology of the Murine Gastrointestinal Tract,” *Infect. Immun.*, vol. 76, no. 3, pp. 907–915, Mar. 2008.

[62] C. G. Buffie and E. G. Pamer, “Microbiota-mediated colonization resistance against intestinal pathogens,” *Nat. Rev. Immunol.*, vol. 13, no. 11, pp. 790–801, Nov. 2013.

[63] L. R. Muniz, C. Knosp, and G. Yeretssian, “Intestinal antimicrobial peptides during homeostasis, infection, and disease,” *Front. Immunol.*, vol. 3, Oct. 2012.

[64] G. O. Canny and B. A. McCormick, “Bacteria in the Intestine, Helpful Residents or Enemies from Within?,” *Infect. Immun.*, vol. 76, no. 8, pp. 3360–3373, Aug. 2008.

[65] M. Suar *et al.*, “Virulence of Broad- and Narrow-Host-Range Salmonella enterica Serovars in the Streptomycin-Pretreated Mouse Model,” *Infect. Immun.*, vol. 74, no. 1, pp. 632–644, Jan. 2006.

[66] O. Gal-Mor, E. C. Boyle, and G. A. Grassl, “Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ,” *Front. Microbiol.*, vol. 5, Aug. 2014.

[67] L. L. Waldner, K. D. MacKenzie, W. Köster, and A. P. White, “From Exit to Entry: Long-term Survival and Transmission of Salmonella,” *Pathogens*, vol. 1, no. 2, pp. 128–155, Oct. 2012.

[68] E. Morgan *et al.*, “Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium,” *Mol. Microbiol.*, vol. 54, no. 4, pp. 994–1010, Nov. 2004.

[69] H. Schmidt and M. Hensel, “Pathogenicity Islands in Bacterial Pathogenesis,” *Clin. Microbiol. Rev.*, vol. 17, no. 1, pp. 14–56, Jan. 2004.

[70] M. Hensel, “Evolution of pathogenicity islands of Salmonella enterica,” *Int. J. Med. Microbiol.*, vol. 294, no. 2, pp. 95–102, Sep. 2004.

[71] M. Raffatellu *et al.*, “SipA, SopA, SopB, SopD, and SopE2 Contribute to Salmonella enterica Serotype Typhimurium Invasion of Epithelial Cells,” *Infect. Immun.*, vol. 73, no. 1, pp. 146–154, Jan. 2005.

[72] L. A. Knodler, J. Celli, W.-D. Hardt, B. A. Vallance, C. Yip, and B. B. Finlay, “Salmonella effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems,” *Mol. Microbiol.*, vol. 43, no. 5, pp. 1089–1103, Mar. 2002.

[73] E. H. Weening, J. D. Barker, M. C. Laarakker, A. D. Humphries, R. M. Tsolis, and A. J. Bäumler, “The Salmonella enterica Serotype Typhimurium lpf, bcf, stb, stc, std, and sth Fimbrial Operons Are Required for Intestinal Persistence in Mice,” *Infect. Immun.*, vol. 73, no. 6, pp. 3358–3366, Jun. 2005.

[74] A. Humphries, S. DeRidder, and A. J. Bäumler, “Salmonella enterica Serotype Typhimurium Fimbrial Proteins Serve as Antigens during Infection of Mice,” *Infect. Immun.*, vol. 73, no. 9, pp. 5329–5338, Sep. 2005.

[75] A. M. Prouty, W. H. Schwesinger, and J. S. Gunn, “Biofilm Formation and Interaction with the Surfaces of Gallstones by Salmonella spp.,” *Infect. Immun.*, vol. 70, no. 5, pp. 2640–2649, May 2002.

[76] A. Fàbrega and J. Vila, “Salmonella enterica Serovar Typhimurium Skills To Succeed in the Host: Virulence and Regulation,” *Clin. Microbiol. Rev.*, vol. 26, no. 2, pp. 308–341, Apr. 2013.

[77] M. Islam, J. Morgan, M. P. Doyle, S. C. Phatak, P. Millner, and X. Jiang, “Persistence of Salmonella enterica serovar typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water,” *Foodborne Pathog. Dis.*, vol. 1, no. 1, pp. 27–35, 2004.

[78] P. Broz, M. B. Ohlson, and D. M. Monack, “Innate immune response to Salmonella typhimurium, a model enteric pathogen,” *Gut Microbes*, vol. 3, no. 2, pp. 62–70, Mar. 2012.

[79] A. Álvarez-Ordóñez *et al.*, “Salmonella spp. survival strategies within the host gastrointestinal tract,” *Microbiology*, vol. 157, no. 12, pp. 3268–3281, 2011.

[80] D. Becker *et al.*, “Robust Salmonella metabolism limits possibilities for new antimicrobials,” *Nature*, vol. 440, no. 7082, pp. 303–307, Mar. 2006.

[81] V. Liévin-Le Moal and A. L. Servin, “Pathogenesis of Human Enterovirulent Bacteria: Lessons from Cultured, Fully Differentiated Human Colon Cancer Cell Lines,” *Microbiol. Mol. Biol. Rev. MMBR*, vol. 77, no. 3, pp. 380–439, Sep. 2013.

[82] “Antibiotic Resistance Threats in the United States, 2013 | Antibiotic/Antimicrobial Resistance | CDC.” [Online]. Available: https://www.cdc.gov/drugresistance/threat-report-2013/index.html. [Accessed: 03-Jul-2017].

[83] B. Stecher *et al.*, “Salmonella enterica Serovar Typhimurium Exploits Inflammation to Compete with the Intestinal Microbiota,” *PLoS Biol.*, vol. 5, no. 10, Oct. 2007.

[84] I. Sekirov *et al.*, “Salmonella SPI-1-mediated neutrophil recruitment during enteric colitis is associated with reduction and alteration in intestinal microbiota,” *Gut Microbes*, vol. 1, no. 1, pp. 30–41, 2010.

[85] S. E. Winter *et al.*, “Gut inflammation provides a respiratory electron acceptor for Salmonella,” *Nature*, vol. 467, no. 7314, pp. 426–429, Sep. 2010.

[86] P. Thiennimitr *et al.*, “Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 42, pp. 17480–17485, Oct. 2011.

[87] H. K. Eltzschig *et al.*, “ATP Release From Activated Neutrophils Occurs via Connexin 43 and Modulates Adenosine-Dependent Endothelial Cell Function,” *Circ. Res.*, vol. 99, no. 10, pp. 1100–1108, Nov. 2006.

[88] M. S. Malo *et al.*, “Intestinal alkaline phosphatase promotes gut bacterial growth by reducing the concentration of luminal nucleotide triphosphates,” *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 306, no. 10, pp. G826–G838, May 2014.

[89] J.-P. Lallès, “Luminal ATP: the missing link between intestinal alkaline phosphatase, the gut microbiota, and inflammation?,” *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 306, no. 10, pp. G824–G825, May 2014.

[90] A. Cauwels, E. Rogge, B. Vandendriessche, S. Shiva, and P. Brouckaert, “Extracellular ATP drives systemic inflammation, tissue damage and mortality,” *Cell Death Dis.*, vol. 5, no. 3, p. e1102, Mar. 2014.

[91] S. Voedisch *et al.*, “Mesenteric Lymph Nodes Confine Dendritic Cell-Mediated Dissemination of Salmonella enterica Serovar Typhimurium and Limit Systemic Disease in Mice,” *Infect. Immun.*, vol. 77, no. 8, pp. 3170–3180, Aug. 2009.

[92] C. M. A. Khan, “The Dynamic Interactions between Salmonella and the Microbiota, within the Challenging Niche of the Gastrointestinal Tract,” *Int. Sch. Res. Not.*, vol. 2014, Jul. 2014.

[93] L. A. Knodler *et al.*, “Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 41, pp. 17733–17738, Oct. 2010.

[94] P. Malik-Kale, C. E. Jolly, S. Lathrop, S. Winfree, C. Luterbach, and O. Steele-Mortimer, “Salmonella – At Home in the Host Cell,” *Front. Microbiol.*, vol. 2, Jun. 2011.

[95] P. Malik-Kale, S. Winfree, and O. Steele-Mortimer, “The Bimodal Lifestyle of Intracellular Salmonella in Epithelial Cells: Replication in the Cytosol Obscures Defects in Vacuolar Replication,” *PLOS ONE*, vol. 7, no. 6, p. e38732, Jun. 2012.

[96] M. Lara-Tejero and J. E. Galán, “Salmonella enterica Serovar Typhimurium Pathogenicity Island 1-Encoded Type III Secretion System Translocases Mediate Intimate Attachment to Nonphagocytic Cells,” *Infect. Immun.*, vol. 77, no. 7, pp. 2635–2642, Jul. 2009.

[97] L. A. Knodler and O. Steele-Mortimer, “Taking Possession: Biogenesis of the Salmonella-Containing Vacuole,” *Traffic*, vol. 4, no. 9, pp. 587–599, Sep. 2003.

[98] R. A. Murray and C. A. Lee, “Invasion Genes Are Not Required for Salmonella enterica Serovar Typhimurium To Breach the Intestinal Epithelium: Evidence That

Salmonella Pathogenicity Island 1 Has Alternative Functions during Infection," *Infect. Immun.*, vol. 68, no. 9, pp. 5050–5055, Sep. 2000.

[99] L. A. Knodler, V. Nair, and O. Steele-Mortimer, "Quantitative Assessment of Cytosolic Salmonella in Epithelial Cells," *PLOS ONE*, vol. 9, no. 1, p. e84681, Jan. 2014.

[100] V. M. D'Costa *et al.*, "Salmonella Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7," *Cell Rep.*, vol. 12, no. 9, pp. 1508–1518, Sep. 2015.

[101] B. K. Coombes, N. F. Brown, Y. Valdez, J. H. Brumell, and B. B. Finlay, "Expression and Secretion of Salmonella Pathogenicity Island-2 Virulence Genes in Response to Acidification Exhibit Differential Requirements of a Functional Type III Secretion Apparatus and SsaL," *J. Biol. Chem.*, vol. 279, no. 48, pp. 49804–49815, Nov. 2004.

[102] J. J. Gilchrist, C. A. MacLennan, and A. V. S. Hill, "Genetic susceptibility to invasive Salmonella disease," *Nat. Rev. Immunol.*, vol. 15, no. 7, pp. 452–463, Jul. 2015.

[103] Ç. Tükel *et al.*, "Toll-like receptors 1 and 2 cooperatively mediate immune responses to curli, a common amyloid from enterobacterial biofilms," *Cell. Microbiol.*, vol. 12, no. 10, Oct. 2010.

[104] A. T. Gewirtz, T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara, "Cutting Edge: Bacterial Flagellin Activates Basolaterally Expressed TLR5 to Induce Epithelial Proinflammatory Gene Expression," *J. Immunol.*, vol. 167, no. 4, pp. 1882–1885, Aug. 2001.

[105] M. T. Abreu, "Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function," *Nat. Rev. Immunol.*, vol. 10, no. 2, pp. 131–144, Feb. 2010.

[106] L. Eckmann, M. F. Kagnoff, and J. Fierer, "Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry.," *Infect. Immun.*, vol. 61, no. 11, pp. 4569–4574, Nov. 1993.

[107] F.-C. Huang, "Regulation of Salmonella flagellin-induced interleukin-8 in intestinal epithelial cells by muramyl dipeptide," *Cell. Immunol.*, vol. 278, no. 1, pp. 1–9, Jul. 2012.

[108] H. K. de Jong, C. M. Parry, T. van der Poll, and W. J. Wiersinga, "Host–Pathogen Interaction in Invasive Salmonellosis," *PLOS Pathog.*, vol. 8, no. 10, p. e1002933, Oct. 2012.

[109] S. Hapfelmeier *et al.*, "The Salmonella Pathogenicity Island (SPI)-2 and SPI-1 Type III Secretion Systems Allow Salmonella Serovar typhimurium to Trigger Colitis via MyD88-Dependent and MyD88-Independent Mechanisms," *J. Immunol.*, vol. 174, no. 3, pp. 1675–1685, Feb. 2005.

[110] S. Mariathasan *et al.*, "Cryopyrin activates the inflammasome in response to toxins and ATP," *Nature*, vol. 440, no. 7081, pp. 228–232, Mar. 2006.

[111] E. A. Miao and J. V. Rajan, "Salmonella and Caspase-1: A complex Interplay of Detection and Evasion," *Front. Microbiol.*, vol. 2, Apr. 2011.

[112] T. Bergsbaken, S. L. Fink, and B. T. Cookson, "Pyroptosis: host cell death and inflammation," *Nat. Rev. Microbiol.*, vol. 7, no. 2, pp. 99–109, Feb. 2009.

[113] I. Jorgensen and E. A. Miao, "Pyroptotic cell death defends against intracellular pathogens," *Immunol. Rev.*, vol. 265, no. 1, pp. 130–142, May 2015.

[114] L. Franchi, "Role of Inflammasomes in Salmonella Infection," *Front. Microbiol.*, vol. 2, Jan. 2011.

[115] M. E. Sellin, K. M. Maslowski, K. J. Maloy, and W.-D. Hardt, "Inflammasomes of the intestinal epithelium," *Trends Immunol.*, vol. 36, no. 8, pp. 442–450, Aug. 2015.

[116] S. M. Man and T.-D. Kanneganti, "Regulation of inflammasome activation," *Immunol. Rev.*, vol. 265, no. 1, pp. 6–21, May 2015.

[117] L. A. Knodler *et al.*, "Non-canonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens," *Cell Host Microbe*, vol. 16, no. 2, pp. 249–256, Aug. 2014.

[118] R. K. S. Malireddi and T.-D. Kanneganti, "Role of type I interferons in inflammasome activation, cell death, and disease during microbial infection," *Front. Cell. Infect. Microbiol.*, vol. 3, 2013.

[119] M. E. Sellin *et al.*, "Epithelium-Intrinsic NAIP/NLRC4 Inflammasome Drives Infected Enterocyte Expulsion to Restrict Salmonella Replication in the Intestinal Mucosa," *Cell Host Microbe*, vol. 16, no. 2, pp. 237–248, Aug. 2014.

[120] C. Cheminay, D. Chakravortty, and M. Hensel, "Role of Neutrophils in Murine Salmonellosis," *Infect. Immun.*, vol. 72, no. 1, pp. 468–477, Jan. 2004.

[121] C. A. MacLennan *et al.*, "The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children," *J. Clin. Invest.*, vol. 118, no. 4, Mar. 2008.

[122] A. Nakano, E. Kita, and S. Kashiba, “Different Sensitivity of Complement to Salmonella typhimurium Accounts for the Difference in Natural Resistance to Murine Typhoid between A/J and C57BL/6 Mice,” *Microbiol. Immunol.*, vol. 39, no. 2, pp. 95–103, Feb. 1995.

[123] E. N. Gondwe *et al.*, “Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal Salmonella by blood cells in Africans,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 7, pp. 3070–3075, Feb. 2010.

[124] O. Steele-Mortimer, “Infection of Epithelial Cells With Salmonella enterica,” in *Bacterial Pathogenesis*, F. DeLeo and M. Otto, Eds. Humana Press, 2008, pp. 201–211.

[125] J. A. Ibarra *et al.*, “Induction of Salmonella pathogenicity island 1 under different growth conditions can affect Salmonella–host cell interactions in vitro,” *Microbiology*, vol. 156, no. Pt 4, pp. 1120–1133, Apr. 2010.

[126] O. Menashe, E. Kaganskaya, T. Baasov, and S. Yaron, “Aminoglycosides Affect Intracellular Salmonella enterica Serovars Typhimurium and Virchow,” *Antimicrob. Agents Chemother.*, vol. 52, no. 3, pp. 920–926, Mar. 2008.

[127] R. M. Tsolis, M. N. Xavier, R. L. Santos, and A. J. Bäumler, “How To Become a Top Model: Impact of Animal Experimentation on Human Salmonella Disease Research,” *Infect. Immun.*, vol. 79, no. 5, pp. 1806–1814, May 2011.

[128] B. B. Madison, L. Dunbar, X. T. Qiao, K. Braunstein, E. Braunstein, and D. L. Gumucio, “Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine,” *J. Biol. Chem.*, vol. 277, no. 36, pp. 33275–33283, Sep. 2002.

[129] R. Maunoury, S. Robine, E. Pringault, N. Leonard, J. A. Gaillard, and D. Louvard, “Developmental regulation of villin gene expression in the epithelial cell lineages of mouse digestive and urogenital tracts,” *Development*, vol. 115, no. 3, pp. 717–728, Jul. 1992.

[130] G. R. Strohmeier *et al.*, “Surface expression, polarization, and functional significance of CD73 in human intestinal epithelia.,” *J. Clin. Invest.*, vol. 99, no. 11, pp. 2588–2601, Jun. 1997.

[131] L. Antonioli, P. Pacher, E. S. Vizi, and G. Haskó, “CD39 and CD73 in immunity and inflammation,” *Trends Mol. Med.*, vol. 19, no. 6, pp. 355–367, Jun. 2013.

[132] H. Zimmermann, “Extracellular metabolism of ATP and other nucleotides,” *Naunyn. Schmiedebergs Arch. Pharmacol.*, vol. 362, no. 4–5, pp. 299–309, Nov. 2000.

[133] K. Enjyoji *et al.*, “Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation,” *Nat. Med.*, vol. 5, no. 9, pp. 1010–1017, Sep. 1999.

[134] C. Martin, M. Leone, X. Viviand, M. Ayem, and R. Guieu, “High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock,” *Crit. Care Med.*, vol. 28, no. 9, pp. 3198–3202, Sep. 2000.

[135] F. Di Virgilio, “Liaisons dangereuses: P2X7 and the inflammasome,” *Trends Pharmacol. Sci.*, vol. 28, no. 9, pp. 465–472, Sep. 2007.

[136] J. K. Crane, R. A. Olson, H. M. Jones, and M. E. Duffey, “Release of ATP during host cell killing by enteropathogenicE. coli and its role as a secretory mediator,” *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 283, no. 1, pp. G74–G86, Jul. 2002.

[137] R. Mempin, H. Tran, C. Chen, H. Gong, K. Kim Ho, and S. Lu, “Release of extracellular ATP by bacteria during growth,” *BMC Microbiol.*, vol. 13, p. 301, 2013.

[138] T. Weissmüller *et al.*, “PMNs facilitate translocation of platelets across human and mouse epithelium and together alter fluid homeostasis via epithelial cell–expressed ecto-NTPDases,” *J. Clin. Invest.*, vol. 118, no. 11, pp. 3682–3692, Nov. 2008.

[139] V. Francois *et al.*, “Intestinal immunopathology is associated with decreased CD73-generated adenosine during lethal infection,” *Mucosal Immunol.*, vol. 8, no. 4, pp. 773–784, Jul. 2015.

[140] Y. Sumi *et al.*, “Plasma ATP is required for neutrophil activation in a mouse sepsis model,” *Shock Augusta Ga*, vol. 42, no. 2, pp. 142–147, Aug. 2014.

[141] Y. Chen *et al.*, “Purinergic Signaling: A Fundamental Mechanism in Neutrophil Activation,” *Sci. Signal.*, vol. 3, no. 125, p. ra45, Jun. 2010.

[142] M. S. Alam, M. G. Costales, C. Cavanaugh, and K. Williams, “Extracellular Adenosine Generation in the Regulation of Pro-Inflammatory Responses and Pathogen Colonization,” *Biomolecules*, vol. 5, no. 2, pp. 775–792, May 2015.

[143] M. S. Alam *et al.*, “CD73 is expressed by human regulatory T helper cells and suppresses proinflammatory cytokine production and Helicobacter felis-induced gastritis in mice,” *J. Infect. Dis.*, vol. 199, no. 4, pp. 494–504, Feb. 2009.

[144] S. C. Robson, “Role of CD73 and extracellular adenosine in disease,” *Purinergic Signal.*, vol. 7, no. 4, pp. 367–372, Dec. 2011.

[145] N. G. Naydenov *et al.*, “Nonmuscle Myosin IIA Regulates Intestinal Epithelial Barrier *in vivo* and Plays a Protective Role During Experimental Colitis,” *Sci. Rep.*, vol. 6, p. srep24161, Apr. 2016.

[146] D. C. Rio, M. Ares, G. J. Hannon, and T. W. Nilsen, “Purification of RNA using TRIzol (TRI reagent),” *Cold Spring Harb. Protoc.*, vol. 2010, no. 6, p. pdb.prot5439, Jun. 2010.

[147] T. D. Schmittgen and K. J. Livak, “Analyzing real-time PCR data by the comparative CT method,” *Nat. Protoc.*, vol. 3, no. 6, pp. 1101–1108, Jun. 2008.

[148] “Tg(Vil-cre)997Gum-alternate1,” *The Jackson Laboratory*. [Online]. Available: https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:24364,004586.

[149] H. Ryu *et al.*, “Development of Quantitative PCR Assays Targeting the 16S rRNA Genes of Enterococcus spp. and Their Application to the Identification of Enterococcus Species in Environmental Samples.” [Online]. Available: http://aem.asm.org. [Accessed: 05-Aug-2017].

[150] S. Devriese *et al.*, “T84 monolayers are superior to Caco-2 as a model system of colonocytes,” *Histochem. Cell Biol.*, vol. 148, no. 1, pp. 85–93, Jul. 2017.

[151] F. Kukulski *et al.*, “NTPDase1 Controls IL-8 Production by Human Neutrophils,” *J. Immunol.*, vol. 187, no. 2, pp. 644–653, Jul. 2011.

[152] F. Bahrami *et al.*, “Purine-Metabolizing Ectoenzymes Control IL-8 Production in Human Colon HT-29 Cells,” *Mediators Inflamm.*, vol. 2014, 2014.

[153] L. Wu, T. Oshima, H. Fukui, J. Watari, and H. Miwa, “Adenosine triphosphate induces P2Y2 activation and interleukin-8 release in human esophageal epithelial cells,” *J. Gastroenterol. Hepatol.*, vol. 32, no. 7, pp. 1341–1347, Jul. 2017.

[154] L. Smith, “Good planning and serendipity: exploiting the Cre/Lox system in the testis,” *Reproduction*, vol. 141, no. 2, pp. 151–161, Feb. 2011.

[155] K. C. King *et al.*, “Rapid evolution of microbe-mediated protection against pathogens in a worm host,” *ISME J.*, vol. 10, no. 8, pp. 1915–1924, Aug. 2016.

[156] M. Castro, M. Sparo, M. Molina, J. Andino, and M. Manghi, “Enterococcus faecalis CECT7121 Induces Systemic Immunomodulatory Effects and Protects from Salmonella Infection,” *Int. J. Probiotics Prebiotics*, vol. 2, no. 4, pp. 215–224, 2007.

[157] M. A. Molina *et al.*, “Immunostimulatory Effects Triggered by Enterococcus faecalis CECT7121 Probiotic Strain Involve Activation of Dendritic Cells and Interferon-Gamma Production,” *PLoS ONE*, vol. 10, no. 5, May 2015.

[158] M. Sparo, G. Delpech, S. Batisttelli, and J. Á. Basualdo, “Immunomodulatory properties of cell wall extract from Enterococcus faecalis CECT7121,” *Braz. J. Infect. Dis.*, vol. 18, no. 5, pp. 551–555, Sep. 2014.

[159] V. K. Ridaura *et al.*, “Gut microbiota from twins discordant for obesity modulate metabolism in mice,” *Science*, vol. 341, no. 6150, p. 1241214, Sep. 2013.

[160] B. M. Brinkman *et al.*, “Gut microbiota affects sensitivity to acute DSS-induced colitis independently of host genotype,” *Inflamm. Bowel Dis.*, vol. 19, no. 12, pp. 2560–2567, Nov. 2013.

[161] M. Gagnon, A. Zihler Berner, N. Chervet, C. Chassard, and C. Lacroix, “Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion,” *J. Microbiol. Methods*, vol. 94, no. 3, pp. 274–279, Sep. 2013.

[162] D. Zhou, L.-M. Chen, L. Hernandez, S. B. Shears, and J. E. Galán, “A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization,” *Mol. Microbiol.*, vol. 39, no. 2, pp. 248–260, Jan. 2001.

[163] M. A. Bakowski, J. T. Cirulis, N. F. Brown, B. B. Finlay, and J. H. Brumell, “SopD acts cooperatively with SopB during Salmonella enterica serovar Typhimurium invasion,” *Cell. Microbiol.*, vol. 9, no. 12, pp. 2839–2855, Dec. 2007.

[164] K. Uchiya, M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman, “A Salmonella virulence protein that inhibits cellular trafficking.,” *EMBO J.*, vol. 18, no. 14, pp. 3924–3933, Jul. 1999.

[165] C. R. Beuzón *et al.*, “Salmonella maintains the integrity of its intracellular vacuole through the action of SifA,” *EMBO J.*, vol. 19, no. 13, pp. 3235–3249, Jul. 2000.

[166] J. Ruiz-Albert, X.-J. Yu, C. R. Beuzón, A. N. Blakey, E. E. Galyov, and D. W. Holden, “Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane,” *Mol. Microbiol.*, vol. 44, no. 3, pp. 645–661, May 2002.

[167] B. Stecher, A. J. Macpherson, S. Hapfelmeier, M. Kremer, T. Stallmach, and W.-D. Hardt, “Comparison of Salmonella enterica Serovar Typhimurium Colitis in Germfree Mice and Mice Pretreated with Streptomycin,” *Infect. Immun.*, vol. 73, no. 6, pp. 3228–3241, Jun. 2005.

[168] I. Sekirov *et al.*, “Antibiotic-Induced Perturbations of the Intestinal Microbiota Alter Host Susceptibility to Enteric Infection,” *Infect. Immun.*, vol. 76, no. 10, pp. 4726–4736, Oct. 2008.