The role of zinc in shaping host-microbe interactions in the gut

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This dissertation by Sarah Rose Gordon is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Publications

1. Taylor, J.R., Wood, J.G., Mizerak, E., Hinthorn, S., Liu, J., Finn, M., **Gordon, S**., Zingas, L., Chang, C., Klein, M.A. and Denu, J.M., **2022**. Sirt6 regulates lifespan in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *119*(5), p.e2111176119.

2. Iyer, N., Grizotte-Lake, M., Duncan, K., **Gordon, S.R**., Palmer, A.C., Calvin, C., Zhong, G., Isoherranen, N. and Vaishnava, S., **2020**. Epithelium intrinsic vitamin A signaling co-ordinates pathogen clearance in the gut via IL-18. *PLoS Pathogens*, *16*(4), p.e1008360.

3. Taylor, J., Wood, J., Finn, M., Liu, J., Mizerak, E., **Gordon, S**., Hutfilz, C. and Helfand, S., **2020**. Mechanisms of Lifespan Extension by Sirt6 in Drosophila melanogaster. *Innovation in Aging*, *4*(Suppl 1), p.736.

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5. Baruah, A., Chang, H., Hall, M., Yuan, J., **Gordon, S**., Johnson, E., Shtessel, L.L., Yee, C., Hekimi, S., Derry, W.B. and Lee, S.S., **2014**. CEP-1, the Caenorhabditis elegans p53 homolog, mediates opposing longevity outcomes in mitochondrial electron transport chain mutants. *PLoS genetics*, *10*(2), p.e1004097.

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Chapter One:

Introduction

Overview:

The gut microbiome is a complex ecosystem, comprising bacteria, fungi, archaea, viruses and helminths, that have evolved to withstand the hostile and competitive conditions within the intestine ((Chasapis et al., 2012, 2020; Lopez & Skaar, 2018c; Ohashi & Fukada, 2019). To survive in the gut, microbes must evade host-imposed immune responses as well as acquire vital nutrients from the host. The transition metal, Zinc (Zn2+), is an essential micronutrient, underlying fundamental molecular and cellular processes in all domains of life (Chasapis et al., 2012, 2020; Lopez & Skaar, 2018c; Ohashi & Fukada, 2019; Xia et al., 2021b). Cellular dysregulation of transition metals, including zinc, can lead to DNA damage, oxidative stress, and cell death(Couñago et al., 2014; Dai et al., 2021; Devisscher et al., 2014a; Kimura & Kambe, 2016; McDevitt et al., 2011; Xu et al., 2019). Due to the necessity and toxicity of zinc, host and gut microbes must compete for zinc in the intestinal lumen, as well as tightly regulate their intracellular zinc concentrations.

The host-microbe interface in the intestine is a harsh environment, where competition for control of transition metals intensifies (Hood & Skaar, 2012; Kehl-Fie et al., 2011; Raffatellu et al., 2009). The host can release of a subset of antimicrobial peptides that chelate metals, such as zinc, manganese and iron, to starve invading pathogens(Besold et al., 2017; Healy et al., 2021; Kehl-Fie et al., 2011; Kehl-Fie & Skaar, 2010). Due to their proximity to the host, members of the mucosal microbial community must employ strategies to endure fluctuations in metal ion availability. To evade host-imposed metal starvation, some colonizers of the mucosal microbiota have evolved high-

affinity transporter systems to acquire and buffer intracellular metal ion concentrations, as well as their own set of metallophores, that sequester ions from the host and other microbes (Elhenawy et al., 2021; Nash et al., 2010).

Despite the hostile environment at the host-microbe interface, unique members of the gut microbiota have evolved to survive in direct contact with the intestinal epithelium. These adherent microbes attach to the intestinal epithelium and drive inflammation in the intestine via accumulation of Th17 cells(Glasser et al., 2001; Ivanov et al., 2009; C.-L. N. Small et al., 2013). Th17 cells secrete pro-inflammatory cytokine IL-17 and are contributors of autoimmunity throughout the body(Bradley et al., 2017a; Talham et al., 1999; Teng et al., 2016).

Expansion of adherent microbes at the intestinal epithelial surface is a common feature found in many patients with Crohn's Disease, a subset of IBD(Baumgart et al., 2007; Shaler et al., 2019) (**Figure 1**). Adherent-invasive *E. coli* (AIEC) induce a potent Th17 cell response and were shown to be enriched in patients with CD(J. G. Lee et al., 2019; Nadalian et al., 2021; C.-L. N. Small et al., 2013). Current efforts to limit Th17 activity in autoimmune diseases focus on the development of drugs that neutralize IL-17(Beringer et al., 2016). However, these efforts have adverse off-target effects in the gut, including increased susceptibility to infection(Fauny et al., 2020). Therefore, identifying strategies to regulate levels of adherent microbes that drive Th17 inflammation in the gut may provide therapeutic relief to IBD patients.

For my thesis, I chose to focus on elucidating the role of zinc in the preservation of adherent microbes at the host-microbe interface. The rapid emergence of antibiotic-resistant bacteria and the growing cost of healthcare has led many, like myself, to search

for alternative, accessible strategies to improve human health. My thesis work provides evidence for the use of zinc supplementation as a potential nutritional approach for dampening inflammatory responses in the gut through depletion of adherent pathobionts.

Zinc is required for all domains of life

Transition metals, including zinc, manganese and iron, are essential for the survival across the animal kingdom, making these metals highly sought after in the intestine(Xia et al., 2021b). The conserved importance of transition metals is due to their incorporation as structural and catalytic component in a vast array of proteins and enzymes(Andreini et al., 2008). In humans, transition metals such as zinc influence many cellular processes, as well as antioxidant and immune responses(Marreiro et al., 2017; Prasad, 2014; Roshanravan et al., 2015). In bacteria, they alter gene expression and protein function, ultimately impacting survival and pathogenicity(Lopez & Skaar, 2018a). Therefore, coordination of transition metal homeostasis is essential for survival of gut bacteria and their host.

Zinc is an essential trace metal for bacteria, impacting their survival and physiology(Lopez & Skaar, 2018b). Zinc can stabilize a diverse array of Zn-finger proteins and are incorporated into enzymes as a catalytic co factor. Alterations in zinc status in bacteria can impact biofilm formation, antibiotic sensitivity, and virulence(Velasco et al., 2018; Xia et al., 2021a). Exposure to high concentrations of zinc can also be toxic to microbes, with studies showing that excess environmental zinc can disrupt cellular maintance of other essential metals.(Couñago et al., 2014; McDevitt et al., 2011; Xu et al., 2019). In *S. pneumoniae,* excess zinc can compete for binding to Mn-transporter substrate, PsaA, leading to manganese starvation(Couñago et al., 2014; McDevitt et al.,

2011). This zinc-imposed starvation of manganese can be directly toxic and can also lead to increase susceptibility to oxidative stress (Eijkelkamp et al., 2014; McDevitt et al., 2011).

Zinc is also important for host immune and inflammatory responses, in main part due to the essential role of zinc as a structural and catalytic component of over ~3,000 human proteins. (Andreini et al., 2006, 2008; Chasapis et al., 2012; Subramanian Vignesh & Deepe, 2017). The regulation of basic cellular processes, such as DNA synthesis and repair, cellular proliferation, differentiation, and apoptosis by zinc influences global immune responses. Zinc can also directly regulate immune processes, including NF-kB signaling and production of antimicrobial peptides(Chyan et al., 2014; Kitabayashi et al., 2010; Podany et al., 2016; Prasad, 2008). Zinc has important roles in regulating barrier permeability and mucosal immune responses in the gut(Foligné et al., 2020; Iwaya et al., 2011; Ohashi & Fukada, 2019). Changes in dietary zinc can induce shifts in the gut microbiota and increase risk for intestinal disease and infection(Foligné et al., 2020; Hibberd et al., 2017; Iwaya et al., 2011; Zackular et al., 2016; Zackular & Skaar, 2018). Dietary zinc deficiency has been linked to host susceptibility to IBD. Moreover, many patients with Crohn's Disease often develop zinc deficiency, which is associated with high levels of inflammation and poor clinical outcomes(Siva et al., 2017; SOLTANI et al., 2021; Vagianos et al., 2007). Zinc supplementation was shown to have protective, anti-inflammatory effects and can improve intestinal barrier function in patients with CD (Ananthakrishnan et al., 2015; Sturniolo et al., 2001), and in mouse models of IBD (Foligné et al., 2020).

Control of zinc at the host-microbe interface

Levels of zinc at the intestinal mucosa are in a dynamic state of flux. Zinc absorption is characterized by a continuous process of uptake of exogenous (dietary) zinc, excretion of endogenous zinc back into the lumen and reabsorption of endogenous zinc(Maares & Haase, 2020). In humans and rodents, zinc absorption is mainly coordinated at the cellular level via enterocytes in the small intestine(Davies, 1980; H. H. Lee et al., 1989). Cellular zinc homeostasis is regulated by transporter families, ZIP and ZnT, and low molecular weight proteins, Metallothionein 1 and 2. ZIP and ZnT control zinc influx or efflux into IECs, while MT1/2 bind zinc at low affinity to buffer levels of intracellular, labile zinc ions(Kimura & Kambe, 2016). Most cellular zinc is tightly bound to proteins and membranes, making these ions less available for utilization by pathogens and gut microbes that interact directly with host IECs(Bozym et al., 2006; Colvin et al., 2008; Li & Maret, 2009). Therefore, the coordination of zinc homeostasis in IEC, which involves constant acquisition and secretion of luminal zinc, changes the availability of this metal to gut microbes.

The host can also regulate metal ion availability in the intestinal lumen to prevent infection. The host has evolved mechanisms, called nutritional immunity, which regulate the availability of nutrients to restrict or aid in microbial growth. While most studies mainly focus on iron sequestration to limit bacterial growth in the gut, several studies indicate that zinc limitation and toxicity is also involved in nutritional immunity(Kehl-Fie & Skaar, 2010). Zinc toxicity, achieved by the release of zinc from intracellular stores, can rapidly kill microbes(Healy et al., 2021). The discovery of calprotectin, a protein heterodimer secreted at mucosal sites that chelates zinc and manganese ions, demonstrates a role of

zinc sequestration in nutritional immunity in the gut(Besold et al., 2017; Kehl-Fie et al., 2011; Liu et al., 2012). Interestingly, MT1/2 are stress-response proteins upregulated and secreted by gut immune cells during inflammation and infection, indicating a potential role of MT1/2 in regulating accessible zinc at the host-microbe interface (Devisscher et al., 2014b). While engaging in nutritional immunity can be effective to prevent infection, host-imposed metal starvation and toxicity may have detrimental effects on commensal microbes, especially those that exist closest to the intestinal epithelium.

To combat the constant changes in metal ion availability at the host-microbe interface, enteropathogens and certain gut microbes have evolved strategies to sequester, acquire and buffer metal ions with high affinity(Flo et al., 2004; Nash et al., 2010; Raffatellu et al., 2009). This includes the use of siderophores, which are high-affinity, small molecular weight proteins that are secreted by bacteria to sequester iron from the host(Page, 2019). Moreover, bacteria have also evolved high-affinity ABC-type transport systems to uptake and buffer cellular metal concentrations(Nash et al., 2010). These transport systems, which contain a substrate binding protein (SBP) that scavenges either zinc, iron or manganese with high affinity, were shown to be susceptible to mismetallation under conditions of zinc excess(Couñago et al., 2014; McDevitt et al., 2011, 2011). In the lung pathogen, *S. pneumoniae*, Zn ions can competitively and irreversibly bind the SBP of the Mn- specific ABC transporter when zinc levels are high, thereby inhibiting Mn uptake(Couñago et al., 2014; Eijkelkamp et al., 2014; McDevitt et al., 2011). This suggests that gut bacteria harboring Mn-ABC transporters may be more susceptible to zinc toxicity, either induced by host immunity or through dietary supplementation.

Adherent gut microbes at the host-microbe interface

The host employs numerous immune strategies to keep gut microbes away from the intestinal epithelium, including release of antimicrobial peptides and mucin production(Duerkop et al., 2009; Lueschow & McElroy, 2020; Okumura & Takeda, 2018; Vaishnava et al., 2008, 2011). Unique members of the gut microbiota can elude host immunity and attach to the intestinal epithelium(Atarashi et al., 2015; Darfeuille-Michaud et al., 2004; Farkas et al., 2015). These adherent microbes are responsible for the induction of T-helper 17 (Th17) cells in the ileum(Atarashi et al., 2015; Farkas et al., 2015; Leonardi et al., 2022; C.-L. N. Small et al., 2013). While these adherent microbes are found within the gut of healthy humans, their expansion within the mucosal microbial community has been implicated in potentiating intestinal inflammation underlying IBD(Chervy et al., 2020a; Darfeuille-Michaud et al., 2004; Glasser et al., 2001; McPhee et al., 2014). Moreover, gut-derived Th17 cells can contribute to inflammation in extra-intestinal sites (Bradley et al., 2017a; Y. K. Lee et al., 2011), suggesting a pivotal role of adherent gut microbes as inducers of autoimmunity throughout the body.

Notably, adherent microbes are distinct from enteropathogens that can similarly attach to epithelium and upregulate Th17 cell responses. Enteropathogens, like *Citrobacter rodentium*, drive overt intestinal inflammation and tissue damage in previously healthy individuals. In contrast, adherent microbes are found in the intestine of healthy animals and can even have beneficial impacts on host immunity. In mice, adherent microbe, Segmented Filamentous Bacteria (SFB), can induce Th17 cells in the small intestine, which are involved in maintaining barrier function and protecting against enteropathogens(Atarashi et al., 2015; Chung et al., 2012; Ericsson et al., 2014; Farkas

et al., 2015). Th17 cells induced by SFB differ in their location, cytokine profile and metabolism compared to Th17 cells elicited by murine enteropathogen, *Citrobacter rodentium* (Omenetti et al., 2019a). While not strict pathogens, adherent microbes can promote disease progression in susceptible hosts. For example, Th17 cells induced by SFB can become autoreactive in mouse models of autoimmune disease and contribute to disease pathology(Bradley et al., 2017a; Flannigan & Denning, 2018; J.-Y. Lee et al., 2020). In humans, adherent microbes, including *Adherent Invasive E. Coli* (AIEC) and *Candida albicans*, are part of the resident gut microbiota in healthy individuals. Overgrowth of these adherent microbes on the epithelial surface is associated with intestinal disease, and contributes to chronic intestinal inflammation seen in patients with IBD(Bacher et al., 2019; Darfeuille-Michaud et al., 2004; Glasser et al., 2001; Shaler et al., 2019; C. L. Small et al., 2016). Thus, adherent microbes are members of the resident gut microbiota in healthy organisms that have context-dependent pathogenic potential.

Segmented Filamentous Bacteria

Segmented Filamentous Bacteria (SFB) are spore-forming, gram-positive, adherent microbes found in the ileum of many organisms, including mice, pigs and some humans(Chen et al., 2018; Hedblom et al., 2018). SFB are unique members of the murine gut microbiota due to their ability to attach to the intestinal epithelium. Moreover, many of the immunomodulatory functions of SFB are due to their ability to attach to IECs (Atarashi et al., 2015). In mice, SFB often adhere to absorptive epithelial cells, likely to extract nutrients from the host, but have also been shown to attach to antigen-processing M cells(Chase & Erlandsen, 1976; Meyerholz et al., 2002). IECs undergo major

transcriptional alterations upon attachment of SFB that culminate in the differentiation of Th17 cells in the small intestine(Atarashi et al., 2015; Ivanov et al., 2009; Talham et al., 1999). Interestingly, germ free mice are void of Th17 cells, while SPF (specific pathogen free) mice without SFB have very low Th17 levels. Oral gavage with SFB in GF and SPF mice signals the differentiation of Th17 cells from naïve CD4+ T-helper cells in the ileum, indicating that SFBs are the main inducers of Th17 cell differentiation in the murine intestine(Ivanov et al., 2009).

SFB are immunomodulatory gut microbes with context-dependent roles in health and disease. On one hand, SFB provide useful immune education without causing overt inflammation and tissue damage(Ivanov et al., 2009; Omenetti et al., 2019b). However, SFB can also contribute to chronic, covert inflammation that can aggravate autoimmunity in susceptible hosts(Bradley et al., 2017a; Flannigan & Denning, 2018; Teng et al., 2016). Th17 cells induced by SFB were shown to promote barrier function by stimulating antimicrobial production in IECs and increasing levels of IgA-secreting cells. SFB was also shown to protect against enteropathogens, including *Salmonella typhimurium* and *Citrobacter rodentium,* as well as control SFB levels at the intestinal surface(Edelblum et al., 2017; Ivanov et al., 2009; Teng et al., 2016). However, SFB was also shown to trigger autoreactive Th17 cells and exacerbate inflammatory diseases, such as rheumatoid arthritis, autoimmune encephalomyelitis and colitis in mice(Bradley et al., 2017a; Flannigan & Denning, 2018; Stepankova et al., 2007; Teng et al., 2016). For example, in a mouse model of rheumatoid arthritis, presence of SFB in the gut triggers a pool of intestinal Th17 cells that are recruited to the lung via CCL20 and contributes to lung pathology(Bradley et al., 2017a). Therefore, gut-derived Th17 cells induced by SFBs can

be beneficial to host immunity, but can also exacerbate autoimmune diseases in susceptible hosts.

Despite the harsh conditions at the epithelial surface, SFB must attach to the intestinal epithelium to survive and persist in the gut(Atarashi et al., 2015). SFB are typically transmitted as dormant spores from parent to offspring(Hedblom et al., 2018). Once inside the gut, SFB spores germinate into flagellated, intracellular offspring (IOs), which anchor themselves to the apical side of IECs using a hook-like, holdfast structure(Nkamba et al., 2020). *In vitro* studies demonstrate that SFB IOs cannot be cultured independently, and instead must be co-cultured with host IECs(Schnupf et al., 2015). Genome sequencing of SFB strains show these microbes lack enzymes for the biosynthesis of critical amino acids and vitamins, indicating that SFB depend on the host for essential nutrients(Sczesnak et al., 2011). Moreover, SFB isolated from one species cannot survive in the gut of another species, due to impaired attachment of SFB to intestinal epithelium(Ericsson et al., 2014). The host-specificity and auxotrophy of SFB suggests that these microbes likely co-evolved with their hosts to inhabit the epithelial surface(Ericsson et al., 2014; Hedblom et al., 2018).

Upon adherence of SFB, the intestinal epithelium undergoes major alterations in signaling and morphology that are critical in shaping homeostatic interactions between gut microbes and host immunity(Flannigan & Denning, 2018). Intestinal epithelial cells respond to the physical burden of SFB attachment by undergoing cytoskeleton rearrangement and transcriptional reprogramming(Atarashi et al., 2015; Ivanov et al., 2009). Upon attachment of SFB, actin accumulation occurs on the underside of the IO holdfast structure, creating a pedestal-like formation to prevent further

invagination(Jepson et al., 1993). Actin cytoskeleton rearrangement also triggers serum amyloid A (SAA1/SAA2) production in IECs, which is released into the lamina propria to promote differentiation and effector function of Th17 cells(Atarashi et al., 2015). Additionally, vesicles form around the distal end of the IO holdfast structure, which uptake SFB antigens through endocytosis and contribute to generation of SFB-specific Th17 cells(Ladinsky et al., 2019). SFB can also adhere to specialized epithelial cells, called microfold (M) cells, that transport luminal antigens and may also be involved in generating SFB-specific Th17 cells(Kobayashi et al., 2019). Induction of Th17 cells in the small intestine can then stimulate antimicrobial production from IECs and increase levels of IgA producing plasma cells, thereby promoting barrier function and spatial organization of the gut microbiota(Chung et al., 2012; Lécuyer et al., 2014). Therefore, IECs are responsible for triggering multiple host signaling pathways upon SFB adherence that are involved in generating Th17 cells and maintaining mucosal homeostasis.

Adherent Invasive E. Coli (AIEC) in Crohn's Disease

To date, there is no single causative agent for the development of Crohn's Disease (CD), a subset of IBD in humans. CD is characterized by inflammation that mainly affects the small intestine, but can also be found anywhere along the GI tract. A combination of factors, including genetics, diet, infection and intestinal microbiota are believed to contribute to the etiology and progression of CD (Agus et al., 2014; Chervy et al., 2020a). Major hallmarks of IBD include loss of intestinal barrier function and microbiota dysbiosis that favors a relative increase in *Proteobacteria,* which includes *Escherichia, Salmonella, Helicobacter* and other gram-negative microbes (Vester-Andersen et al., 2019).

Disruption of the gut barrier leads to bacterial adherence and translocation into host tissue, which drives intestinal inflammation (J. G. Lee et al., 2019; Ohashi & Fukada, 2019; Okumura & Takeda, 2018; Sturniolo et al., 2001).

AIEC are members of the human gut microbiota that can attach to and invade the intestinal epithelium(Barnich et al., 2003; Chervy et al., 2020b; Palmela et al., 2018; Sevrin et al., 2020; Yang et al., 2017). The overgrowth of AIEC at the epithelial surface has been implicated in both the etiology and progression of CD(Chervy et al., 2020a; Shaler et al., 2019). The prevalence, abundance and richness of AIEC in the ileal mucosa are greater in patients with CD compared controls(Darfeuille-Michaud et al., 2004; Nadalian et al., 2021). Moreover, expansion of AIEC correlates with increased inflammatory burden seen in patients with CD(Chervy et al., 2020a; Glasser et al., 2001; Yang et al., 2017). Studies in mice demonstrate that AIEC can attach to intestinal epithelium and induce pro-inflammatory Th17 and Th1 cell responses, indicating a role of AIEC in driving chronic intestinal inflammation(C.-L. N. Small et al., 2013).

AIEC are found in otherwise healthy individuals, indicating they do not act independently to induce disease or infection. Instead, AIEC likely work in concert with other environmental and/or genetic stressors to drive CD. Most strains of *E. coli* can be categorized using a combination of genetics, phylogeny and clinical outcomes as either commensal or overtly pathogenic(Chassaing & Darfeuille-Michaud, 2013; Martinez-Medina et al., 2009; McPhee et al., 2014; Shaler et al., 2019). However, AIEC do not fall neatly into either category and their role in disease appears to be context-dependent. AIEC are distinct from other pathogenic *E coli,* including the six major classes of diarrheagenic *E. coli* pathotypes, as they do not express common virulence factors(Nash

et al., 2010; Shaler et al., 2019). Genomic studies of AIEC could not identify a clear genetic basis for their pathogenic phenotype found *in vivo(Nash et al., 2010).* Instead, AIEC appear to have acquired genes often found in enteropathogens that confer competitive fitness advantage, particularly within the inflamed intestine (Nash et al., 2010). For example, AIEC contain genes involved in AMP resistance, enhanced acquisition of iron and other transition metals, regulation of flagella, and utilization of alternative carbon sources (Chassaing & Darfeuille-Michaud, 2013; Martinez-Medina et al., 2009; McPhee et al., 2014; Nash et al., 2010). These adaptations, among many others, allow AIEC to outcompete commensal microbes, gain access to the epithelial surface and thrive in the inflamed gut. Therefore, AIEC behaves as an opportunistic pathobiont capable of potentiating CD.

Adherent microbes are unique compared to other commensals due to their ability to bypass host immunity, including mucus and AMPs, to attach to the intestinal epithelium(Barnich et al., 2003; McPhee et al., 2014; Nash et al., 2010; Sevrin et al., 2020; C. L. Small et al., 2016). AIEC, like SFB, utilize flagella to traverse the mucus layer and gain access to the epithelial surface(Barnich et al., 2003; Sevrin et al., 2020). Unlike other commensal *E. coli* strains, AIEC were shown to upregulate flagellum expression in the presence of mucus, which provides AIEC a selective advantage at the intestinal mucosa(Barnich et al., 2003; Sevrin et al., 2020). Moreover, AIEC can finely regulate their flagellum production, which aids in immune evasion(Sevrin et al., n.d.). AIEC strains have also evolved mechanisms to evade host AMP responses, which allows them to survive at the epithelial surface(McPhee et al., 2014; Nash et al., 2010; C. L. Small et al., 2016).

AIEC strain NRG857c contains multiple genes that confer AMP resistance, including an outer membrane protease (Nash et al., 2010).

Physiological changes within the inflamed intestine can provide a selective advantage favoring colonization and expansion of AIEC in the intestinal mucosa which is the main factor influencing their pathogenic potential (Kitamoto et al., 2020; McPhee et al., 2014; Sevrin et al., 2020; C. L. Small et al., 2016), (Agus et al., 2014; Baumgart et al., 2007; Duerkop et al., 2009; Hooper et al., 2012; Palmela et al., 2018; C. L. Small et al., 2016). Upregulation of host AMP production upon infection with enteropathogens, such as *C diff* and STM, drives AIEC expansion, thereby exacerbating disease severity(C. L. Small et al., 2016). Moreover, the opportunistic bloom of AIEC in response to infection was dependent on bacterial genes that confer defensin resistance (C. L. Small et al., 2016). These same immune responses often become upregulated in CD patients, which allows AIEC to outcompete commensal microbes and thrive in the intestinal mucosa (Barnich et al., 2003; McPhee et al., 2014; C. L. Small et al., 2016).

Antibiotic use may further enrich for AIEC colonization and overgrowth within the gut. AIEC genome contains many genes responsible for resistance to antibiotics, such as tetracycline, chloramphenicol and beta-lactams (Nash et al., 2010). Moreover, AIEC can produce robust biofilms, which could increase survival of AIEC at the epithelial surface upon antibiotic treatment (Martinez-Medina et al., 2009).

To survive in the inflamed gut, AIEC have acquired mechanisms typically used by pathogens to survive in close contact with the host. In addition to AMP resistance and flagella regulation, AIEC have also evolved ways to combat nutritional immunity posed by the host. AIEC strains, including NRG857c, were shown to contain high-affinity

transporter systems for trace metals, including iron, zinc and manganese(Nash et al., 2010). AIEC has also acquired high-affinity manganese transporters, including sitABC, which typically transports Mn2+ but can also transport Fe2+(Nash et al., 2010). The uptake of manganese via sitABC is a conserved strategy used by other invasive pathogens to combat the high levels of oxidative stress encountered during close contact with the host(McDevitt et al., 2011). These transition metals are often used by bacteria as co-factors for enzymes involved in the protection against oxygen toxicity and utilization of alternative carbon sources. Indeed, the metabolic fitness of AIEC within the mucosa was shown to be regulated by enzymes that rely on iron as a co-factor (Elhenawy et al., 2021; Kitamoto et al., 2020). Siderophores, which are molecules typically secreted by enteropathogens to sequester iron, are also expressed by AIEC. NRG857c was shown to upregulate expression of yersiniabactin, a siderophore which can escape host-imposed lipcalin-2 sequestration (Elhenawy et al., 2021).

Interestingly, AIEC strains are genetically diverse, which suggests that there were different founder populations that gave rise to this pathobiont type (Shaler et al., 2019). This indicates that genetic and environmental factors shaping the inflamed gut could potentially act as evolutionary pressures giving rise to new AIEC strains as well as increasing abundance of previously existing AIEC strains within the mucosal community (Chervy et al., 2020a).

Regulation of Th17 cells via gut microbes

T-helper 17 (Th17) cells are a subset of T-helper cell and are major regulators of mucosal homeostasis in the gut by influencing epithelial cell responses and protecting

against enteropathogens (Farkas et al., 2015; Lai et al., 2020). However, dysregulation of Th17 cell responses is implicated in the progression and severity of many autoimmune diseases (Bradley et al., 2017b, 2017a; Healy et al., 2021). Th17 cells are distinguished from other CD4+ T-helper cells due to their expression of transcription factor, RORgt, and their production of IL-17A, IL-17F and IL-22 (Wan, 2010). Studies in mice demonstrate that the major regulators of Th17 induction in the intestine are adherent gut microbes (Farkas et al., 2015; Ivanov et al., 2009). Moreover, interactions between gut microbes, epithelial cells, antigen presenting cells (APCs), and other T-helper cells are involved in directing and controlling Th17 cell activity in the gut (Omenetti & Pizarro, 2015; Smith et al., 2013; Yan et al., 2020).

Induction of Th17 cells occurs via adherent microbes

Segmented Filamentous Bacteria (SFB), as well as other adherent microbes, such as *Candida albicans* and AIEC, induce potent Th17 cell responses in the gut(Bacher et al., 2019; Ivanov et al., 2009; C.-L. N. Small et al., 2013). Germ free mice are void of Th17 cells and Jackson mice, which do not contain SFB, have very low levels of Th17 cells(Farkas et al., 2015). In contrast, Taconic mice, which have SFB as part of their resident gut microbiota, show potent Th17 cell induction in the small intestine(Farkas et al., 2015; Ivanov et al., 2009; Omenetti et al., 2019a). Oral gavage with SFB in GF and Jackson mice signals the generation of Th17 cells from naïve CD4+ T-helper cells in the ileum(Ivanov et al., 2009). Moreover, colonization with SFB strains that cannot attach to the intestinal epithelium abrogates Th17 induction, indicating that adherence to IECs is critical for Th17 cell induction in the gut(Atarashi et al., 2015).

When SFB attaches to the intestinal epithelium, IECs undergo major transcriptional and morphological changes that direct Th17 cell differentiation and effector function in the small intestine (Atarashi et al., 2015; Ivanov et al., 2009). SFB attachment triggers IEC production of SAA1 and SAA2, which are acute phase proteins typically expressed during infection. SAAs are secreted from IECs into the lamina propria, where they act on primed RORgT+ Th17 cells in to stimulate IL-17A and IL-17F production (J.-Y. Lee et al., 2020). *In vitro* studies demonstrate that different combinations of SAA, IL-6, IL-23, TGFb, IL-21, IL-1B, and other cytokines, can induce Th17 cell differentiation (Bettelli et al., 2006; Ghoreschi et al., 2010; J.-Y. Lee et al., 2020; Manel et al., 2008; Sano et al., 2021). These finding, along with *in vivo* studies, suggest that there are likely redundant cytokine signals produced by IECs and other immune cells that guide Th17 cell differentiation.

The priming, differentiation and maturation of Th17 cells via the gut microbiota involves a coordinated response between IECs and APCs. The main location of Th17 cell priming in response to SFB is still debated, though there is evidence that it can occur in the lamina propria, peyer patches, and MLNs (Farkas et al., 2015; Geem et al., 2014; Hirota et al., 2013; Sano et al., 2021). APCs, including DCs and macrophages, acquire SFB antigens and prime naïve CD4+ T cells. APCs may acquire SFB antigens from direct contact with SFB or through interactions with IECs, which were shown to uptake SFB antigens through endocytosis (Ladinsky et al., 2019). Differentiation of Th17 cells from naïve Th cells occurs through Stat3-dependent induction of RORgT. IL-6, IL-23, IL-22 and IL-1B produced by APC promote SFB-directed Th17 cell differentiation. Maturation occurs in the lamina propria where SAA1 and SAA2 produced by epithelial cells act on

RORgT+ Th17, resulting in the production of effector cytokines, IL-17A and IL-17F (J.-Y. Lee et al., 2020).

Certain members of the gut microbiota can control Th17 activity via induction of T-regulatory cells (Tregs)(Omenetti & Pizarro, 2015). T-regulatory cells are a type of T-helper cell that are identified by their expression of FoxP3 and effector cytokines, IL-10 and TGFb(Wan, 2010). Tregs are important for their often immunosuppressive, anti-inflammatory effects in the gut. Imbalance of Th17/Treg responses, in favor of Th17 activity, is a common feature of many inflammatory diseases, including IBD(Yan et al., 2020). *Clostridia* are potent inducers of Treg activity due to their production of short chain fatty acids(Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). Another regulator of Tregs is PSA derived from *B.fagilis* which enhances expression of effector molecules including IL-10(Round et al., 2011). Therefore, members of the gut microbiota can suppress Th17 activity by increasing Treg differentiation and effector function.

Dietary nutrients can impact Th17 activity, either directly by regulating differentiation of naïve CD4+ T cells and/or via changes in the gut microbiota. Whether zinc supplementation *in vivo* impacts th17 activity in the gut has not yet been shown. Zinc excess was shown to alter fecal microbiota in mice, including relative increase in *Clostridiaceae* and decrease in *Candidatus arthromitus,* though the role this has on Th17/Treg balance remains unanswered(Foligné et al., 2020; Zackular et al., 2016). *In vitro* studies indicate that zinc can increase Treg levels*,* and that DCs may be involved in directing Treg differentiation in responses to zinc(George et al., 2016). Zinc was also show to inhibit STAT3, thereby suppressing RORgt+ Th17 cell differentiation *in vitro*(Kitabayashi et al., 2010). For these reasons, many of the protective effects of zinc

supplementation are often attributed to its direct effect on host-intrinsic cellular processes, such as antioxidant responses and T-cell differentiation (Chasapis et al., 2012; Ohashi & Fukada, 2019). Since zinc can also affect gut microbes, it is not known whether some of these anti-inflammatory effects of zinc supplementation are mediated by alterations in the gut microbiota. In my thesis work, I found that zinc supplementation can change the inflammatory potential of the gut microbiota by targeted depletion of adherent pathobionts.

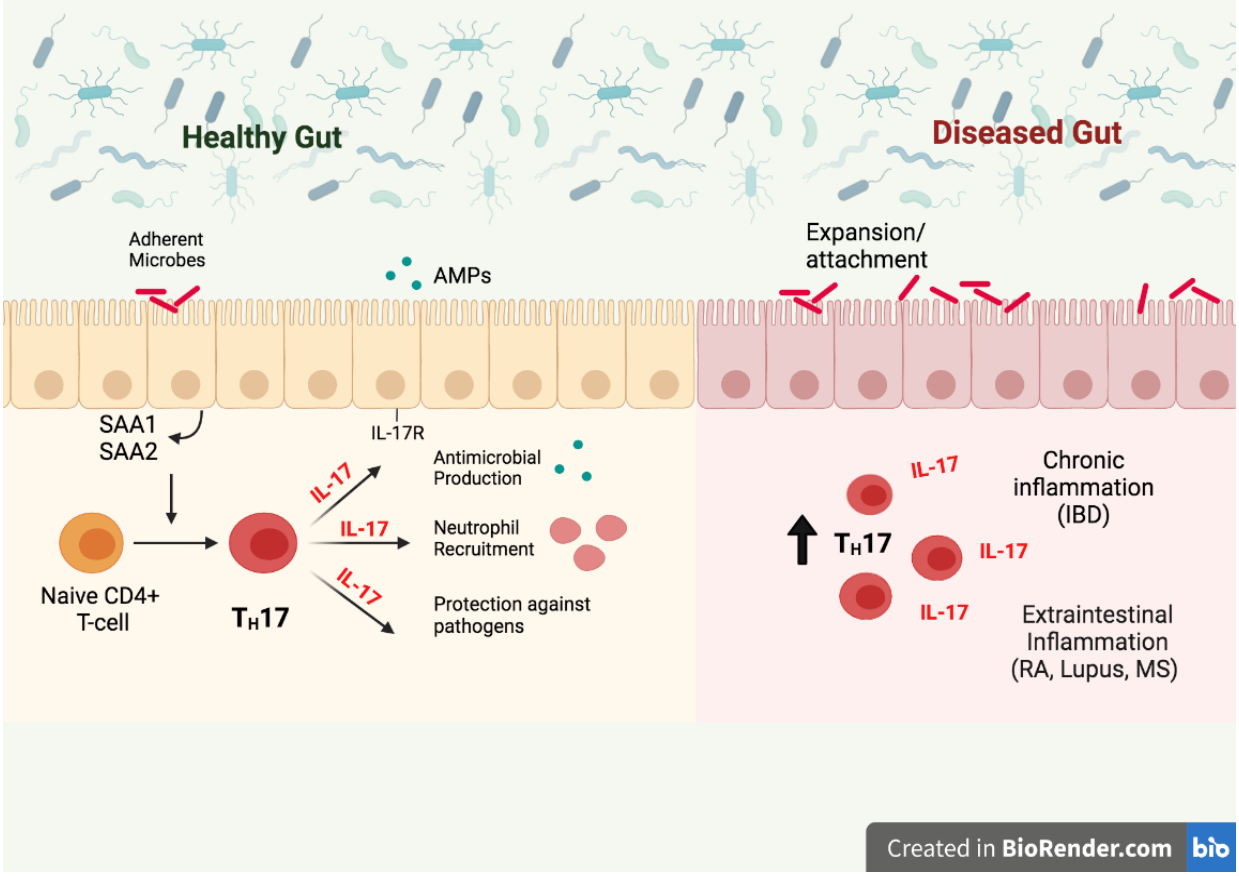

Figure 1: Adherent microbes perpetuate Th17-driven intestinal inflammation Adherent microbes attach to intestinal epithelial cells and induce differentiation of pro-inflammatory Th17 cells in the gut. Th17 cells play important functions in a healthy gut and protect against infection. However, overgrowth of adherent microbes is associated with high levels of Th17-mediated inflammation, which contributes to severity of autoimmune diseases, like Inflammatory Bowel Disease (IBD). Gut-derived Th17 cells can also travel outside the gut, where they can contribute to diseases like Multiple Sclerosis (MS) and Rheumatoid Arthritis (RA).

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Chapter Two

Zinc therapy reduces inflammatory potential of the gut microbiota via targeted depletion of adherent bacteria

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Abstract

Adherent bacteria are members of the resident gut microbiota that can bypass host-imposed immune responses to directly attach to the intestinal epithelium. While adherent bacteria are found within the gut of healthy individuals, their colonization and expansion within the ileal mucosal microbiota exacerbates local and extra-intestinal inflammation underlying various autoimmune diseases, such as Crohn's Disease, Rheumatoid Arthritis, and Multiple Sclerosis. Here, we tested zinc supplementation on two adherent microbes, Segmented Filamentous Bacteria (SFB) and a strain of Adherent-Invasive E coli (AIEC) isolated from the ileal mucosa from a patient with Crohn's Disease. Zinc therapy depleted SFB from the murine mucosal microbiota, thereby dampening intestinal T-helper 17 (Th17) cell induction *in vivo.* Th17 cells induced by SFB, which are involved in maintaining barrier function and protecting against enteropathogens*,* were also shown to become autoreactive and contribute to disease pathology in susceptible hosts. Fecal microbiota transfer (FMT) from zinc treated mice into germ-free mice recaptured the reduced Th17 cell phenotype. Additionally, zinc therapy cleared AIEC from the murine ileal mucosa in a pre-clinical model of AIEC colonization. We found that the antibacterial effect of zinc on AIEC may be due to dysregulation of another key metal, manganese. Manganese (Mn) is an essential micronutrient for bacterial pathogens to combat the high levels of oxidative stress encountered during close contact with their host. Our work provides a novel nutritional approach to dampen inflammatory potential of the gut microbiota through targeted elimination of adherent bacteria from the ileal mucosa.

Introduction

Diet is a major factor that shapes host-microbiota interactions in the gut (Hibberd et al., 2017; Khan et al., 2020; Yoo et al., 2021). Dietary micronutrients (vitamins and minerals) are cofactors for a vast array of enzymes spanning all domains of life, making them highly sought after in the intestine (Hood & Skaar, 2012; Lopez & Skaar, 2018; Xia et al., 2021). In the inflamed gut, trace metals, such as iron, zinc and manganese, become further restricted by the host in a process known as nutritional immunity (Hood & Skaar, 2012). To ward off invading microbes, the host can release of a subset of antimicrobial peptides that chelates iron, zinc or manganese to starve pathogens of these essential metal ions (Flo et al., 2004; Hood & Skaar, 2012; Raffatellu et al., 2009). This includes protein heterodimer, calprotectin (S100A8/S100A9), which sequesters manganese and zinc ions away from pathogens and nearby gut microbes (Lopez et al., 2019; Zackular & Skaar, 2018).

To survive in the gut, microbes must evade host-imposed immune responses as well as acquire vital nutrients from the host. The transition metal, zinc, is an essential micronutrient, underlying fundamental molecular and cellular processes in all domains of life (Xia et al., 2021). Cellular dysregulation of transition metals, including zinc, can lead to DNA damage, oxidative stress, and cell death (Lopez & Skaar, 2018; Radin et al., 2019). Due to the necessity and toxicity of zinc, host and gut microbes must compete for zinc in the intestinal lumen, as well as tightly regulate their intracellular zinc concentrations. Previous studies involving zinc excess and deficiency show that zinc can

alter fecal microbial composition (Foligné et al., 2020; Hibberd et al., 2017; Zackular et al., 2016). However, very little attention has been given to how zinc alters distinct microbial communities in the gut and the effect this has on host immunity.

In this study, we identified a novel effect of zinc supplementation in depletion of two adherent microbes within the ileal mucosal community. We show that depletion of adherent microbes reduces inflammatory potential of the gut microbiome. While adherent bacteria are found within the gut of healthy individuals, their colonization and expansion within the ileal mucosal microbiota is implicated in exacerbating local and systemic inflammation underlying various autoimmune diseases, such as Crohn's Disease, Rheumatoid Arthritis, and Multiple Sclerosis (Bradley et al., 2017; Darfeuille-Michaud et al., 2004; Y. K. Lee et al., 2011; Palmela et al., 2018; Vester-Andersen et al., 2019). Here we show that zinc supplementation can alter the inflammatory potential of the gut microbiota by editing-out adherent pathobionts from the ileal mucosa.

Results

Zinc treatment depletes adherent microbe SFB from ileal mucosal community

Around 15-40% of patients with Inflammatory Bowel Disease, which encompasses both Crohn's Disease and Ulcerative Colitis, develop zinc deficiency during their disease(Alkhouri et al., 2013; Vagianos et al., 2007). Zinc deficiency is associated with poor clinical outcomes for patients with IBD (Siva et al., 2017), and is thought to exacerbate intestinal inflammation through disruption of epithelial barrier function and impaired mucosal immunity (Ananthakrishnan et al., 2015; Foligné et al., 2020; Vagianos et al., 2007). For this reason, zinc supplements are prescribed to CD patients, which was

shown to improve barrier integrity and dampen intestinal inflammation (Sturniolo et al., 2001). The protective effects of zinc supplementation in the context of IBD are often attributed to its direct effect on host-intrinsic cellular processes, such as antioxidant responses and T-cell differentiation (Chasapis et al., 2012; Ohashi & Fukada, 2019).

Since zinc can also affect gut microbes, it is not known whether some of these anti-inflammatory effects of zinc supplementation are mediated by alterations in the gut microbiota. To examine the impact of zinc supplementation on host-microbiota interactions in the gut, we treated C57BL/6 (B6) mice (Taconic Farms) with 25mM $ZnSO_4$-$7H_2O$ in their drinking water for 1 week. All mice used in this study were littermates and co-housed prior to zinc treatment. The zinc concentration used in this study was modeled after therapeutic zinc supplementation in humans, and contains approximately 10-fold the zinc level of standard mouse chow (Zackular et al., 2016). Mice that received zinc treatment showed no signs of metal toxicity. Mice provided similar ppm of zinc for similar or longer durations also reported no signs of toxicity (Foligné et al., 2020; Zackular & Skaar, 2018).

Previous studies involving zinc excess and deficiency show that zinc can alter fecal microbial composition (Foligné et al., 2020; Hibberd et al., 2017; Zackular et al., 2016). However, very little attention has been given to how zinc alters distinct microbial communities in the gut and the effect this has on host immunity. To explore the effect of zinc supplementation in the small intestine, luminal microbes of the distal small intestine (SI) were extracted from zinc-treated and control (untreated) mice and 16s rRNA amplicon sequencing was used to assess changes in bacterial community structure (Figure 1A).

Excess zinc can be toxic to microbes by disrupting cellular homeostasis of other essential metals, such as manganese, iron and/or copper (Couñago et al., 2014; McDevitt et al., 2011; Xu et al., 2019). However, zinc treatment did not alter the diversity of the SI luminal microbiota. There was no significant difference in observed amplicon sequence variants (ASVs) from the luminal community of zinc-treated vs. untreated mice, indicating that zinc supplementation did not impact richness of the luminal community (Figure 1B). The Shannon-diversity index that measures both species richness and evenness was also not significantly different between zinc-treated vs. untreated samples (Figure 1C). Taken together, these results indicate that zinc treatment did not alter the alpha diversity of the SI luminal microbiota. Beta diversity was also unchanged, as shown by PCoA plot based on unweighted UniFrac distances (Figure 1D).

By plotting the relative abundances of bacteria at the genus level, we uncovered that the percent relative abundance of SFB (*Candidatus Arthromitus*) in zinc-treated samples was significantly reduced compared to untreated samples (Figure 1E-G). SFB are spore-forming, adherent microbes that colonize the ileum of the small intestine in Taconic mice and contribute to host immune education through their attachment to the intestinal epithelium (Atarashi et al., 2015; Ivanov et al., 2008, 2009). Linear discriminant analysis (LDA) effect size (LEfSe) of luminal microbiota further revealed that *Candidatus Arthromitus* was the only genus significantly enriched in control samples (Figure 1G), suggesting a selective depletion of SFB in the small intestine in response to zinc therapy.

Many of the immunomodulatory functions of SFB occur via its attachment to the ileal epithelium (Atarashi et al., 2015; Sczesnak et al., 2011). To explore the effect of zinc supplementation at the host-microbe interface, the mucosal microbiota of the ileum was

extracted from zinc-treated and untreated mice and 16s rRNA amplicon sequencing was used to assess changes in bacterial community structure (Figure 1H). There was no significant difference in observed amplicon sequence variants (ASVs) from the mucosal community of zinc-treated vs. untreated mice, indicating that zinc supplementation did not impact richness of the mucosal community (Figure 1I). The Shannon-diversity index that measures both species richness and evenness was also not significantly different between zinc-treated vs. untreated samples (Figure 1J). Taken together, these results indicate that zinc treatment did not alter the alpha diversity of the ileal mucosal microbiota.

SFB must attach to IECs to persist in the gut (Atarashi et al., 2015; Sczesnak et al., 2011). We observed that SFB make up a major part (~25% relative abundance) of the ileal mucosal microbiota of Taconic mice and that SFB levels were depleted in the mucosal microbiota of zinc supplemented mice (Figure 1L-N). Using Analysis of Composition of Microbiomes (ANCOM), we found that the most significantly different ASVs between zinc-treated and control mucosal microbiotas were identified as *Candidatus Arthromitus* (Figure 1M), indicating a profoundly sensitivity of SFB to zinc treatment. Many members of the ileal mucosal microbiota have potent immunomodulatory functions due to their proximity to host epithelium (Duerkop et al., 2009; Hooper et al., 2012; Ivanov et al., 2009; Leonardi et al., 2022; Vuik et al., 2019). Compositional changes of the mucosal microbiota have been implicated in the etiology and/or progression of several inflammatory diseases (Bradley et al., 2017; Y. K. Lee et al., 2011; Palmela et al., 2018; Rowan et al., 2010). Our data suggests that zinc supplementation restricts SFB colonization of the ileal mucosal microbiota, which likely prevents its persistence throughout the small intestine.

SFB colonization status is reversed by zinc treatment in conventional and germ free mice

Unlike Taconic mice, Jackson mice do not contain SFB as part of gut microbiome (Farkas et al., 2015a). Colonization of Jackson mice with mouse-specific SFB induces Th17 differentiation in the small intestine (Atarashi et al., 2015; Farkas et al., 2015b; Ivanov et al., 2009). We next colonized Jackson mice with SFB via oral gavage and subjected these SFB+ Jackson mice to zinc treatment (Figure 2A). At three weeks post-colonization, the relative abundance of SFB in the mucosal microbiota of control SFB+ Jackson mice was >50% (Figure 2B). The percent relative abundance of SFB in the mucosal microbiota of zinc-treated SFB+ Jackson mice was significantly reduced compared to untreated controls (Figure 2B-C).

To determine whether microbe-microbe interactions are required for depletion of SFB in response to zinc treatment, we tested the effect of zinc treatment on SFB levels in SFB mono-colonized mice. Briefly, germ- free mice were exposed to fecal content from previously SFB mono-colonized mice and cohoused for three days. Next, mice were placed at random into two cages and supplied either zinc-supplemented or supplemented water (Figure 2D). The ileal mucosal microbiota was extracted after one week. Using 16s rRNA amplicon sequencing, we found that SFB comprised ~100% of the mucosal microbiota in control mice, as expected (Figure 2E). In contrast, SFB was eliminated from the mucosal community (0%) of zinc-treated mice (Figure 2F). Assessment of SFB levels in feces using qPCR further confirmed that mice had minimal detectable SFB DNA after zinc treatment, and SFB mono-colonized mice returned to pre-colonization levels of

bacterial load after zinc treatment indicating that zinc completely abrogates SFB from the gut (Figure 2G).

We also explored the kinetics of SFB depletion in response to zinc treatment. Assessment of SFB DNA in feces using qPCR showed that SFB levels become significantly reduced within the first 24 hours of zinc treatment. By d3 of zinc treatment, SFB DNA levels dropped to those seen at d7 (Figure S1A). These findings demonstrate that short-term zinc treatment (within ~2-3 days) is sufficient for depletion of SFB from the gut microbiota. Innate immune responses, such as antimicrobial peptide production, restrict growth of SFB within the mucosal community. To assess the antimicrobial activity of the ileal mucosa in response to zinc treatment, we performed a bacterial killing assay using mucosal scrapings isolated from zinc-treated and untreated mice (Figure S1B). We found that mucosal content of untreated mice resulted in ~20% bacterial killing compared to PBS controls (Figure S1C). Bacterial killing increased to 46% when bacteria were incubated with mucosal content from zinc-treated mice, indicating that zinc treatment enhanced the antimicrobial activity of ileal mucosa more than two-fold.

Zinc-treatment reduces intestinal Th17 cell population

The attachment of SFB to intestinal epithelium drives differentiation of naïve T-helper cells into T-helper 17 cells in the small intestine (Atarashi et al., 2015). Th17 cells induced by SFB, which are involved in maintaining barrier function and protecting against enteropathogens, were also shown to become autoreactive and contribute to disease pathology in susceptible hosts(Bradley et al., 2017; Flannigan & Denning, 2018; Y. K. Lee et al., 2011). Given the loss of SFB from the gut microbiota of zinc-treated mice, we looked

for signs of diminished Th17 cell activity in these mice. Th17 cells are distinguished from other CD4+ T-helper cells by their expression of *RORgT* and production of IL-17 and IL-22 (Omenetti & Pizarro, 2015). We isolated lymphocytes from the small intestine lamina propria (SI LPL) and used flow cytometry to compare Th17 cell population between zinc-treated and untreated mice. Zinc-treated mice showed a significant reduction in percentage of live CD4+ cells (Figure 3A-B), indicating a reduced T-helper cell population. We did not observe changes in percentage of live CD8+ cells between zinc treated and untreated mice (Figure 3A-B). Zinc treated mice also had a reduced percentage of RORgt and IL-17 expressing T-helper cells (Figure 3C-D), indicating a reduced Th17 cell population in these mice. Overall, our findings suggest loss of SFB from mucosal microbiota of zinc treated mice coincides with loss of intestinal Th17 cells.

Intestinal epithelial cells respond to the physical burden of SFB attachment by undergoing major morphological and transcriptional changes (Ivanov et al., 2009; Talham et al., 1999). SFB attachment triggers upregulation of SAA1 and SAA2 in IECs, which are acute phase proteins typically upregulated during infection (Flannigan & Denning, 2018; J.-Y. Lee et al., 2020). SAAs are secreted from IECs into the small intestine lamina propria, where they help direct Th17 cell differentiation (Atarashi et al., 2015; Ivanov et al., 2009; Sano et al., 2015) (Figure 3E). We found that zinc-treated mice have reduced *saa1* and *saa2* mRNA expression in the small intestine (Figure 3F), as well as reduced SAA-1 expression in IECs (Figure 3G), suggesting that abridged Th17 activity in zinc-treated mice may be due to loss of SFB from the mucosal microbiota.

Zinc-treatment dampens Th17 cell induction through depletion of SFB

To further explore the relationship between zinc, SFB, and Th17 cell activity, we subjected mice to a range of different concentrations of zinc sulfate (5mM, 10mM, 15mM, 25mM and 100mM) for one week. We assessed SFB DNA in feces using qPCR and found that zinc targets SFB in dose-dependent manner (Figure 4A). The concentration-dependent effects of zinc on SFB is independent of altered 16S levels. Furthermore, we observed that diminished SFB levels coincides with significant reduction in *il-17* mRNA expression in the ileum (Figure 4B). We found that a dose of 10mM zinc sulfate significantly reduced SFB and *il-17* levels without complete elimination of SFB from the gut microbiota (Figure 4A-B). This presents the possibility of utilizing different concentrations of zinc to regulate abundance of SFB at the host-microbe interface.

The anti-inflammatory properties of zinc are often attributed to its direct effect of zinc on immune cell differentiation and function(Chasapis et al., 2012, 2020; Ohashi & Fukada, 2019). It has been documented that *in vitro* zinc supplementation can suppress Th17 cell differentiation through altered STAT3 activity (Kitabayashi et al., 2010). However, based on our findings, we wanted to explore the possibility that zinc supplementation could be suppressing Th17 cell induction through changes in the gut microbiota. To determine this, we transferred the microbiota of zinc-treated mice into germ-free mice (Figure 4C). Additionally, we transferred the microbiota of untreated littermates into a second set of germ free mice as our control group. Germ free mice used in both groups received untreated water before and after microbiome transfer. After 14 days, we isolated immune cells from the SI lamina propria and used flow cytometry to compare Th17 cell population between both groups. Colonization with the gut microbiota of zinc-treated mice resulted in significantly less Th17 cell induction compared to

colonization with control microbiota (Figure 4D-E), indicating that loss of SFB from the gut microbiota in response to zinc-treatment significantly dampens Th17 induction *in vivo*.

Zinc therapy reduces AIEC bacterial burden and clears AIEC from ileum

We next wanted to assess whether our zinc treatment could be useful for elimination of adherent microbes associated with human inflammatory bowel disease (IBD). *Adherent-Invasive E. Coli* (AIEC) are members of the human gut microbiota that can attach to and invade the intestinal epithelium (Darfeuille-Michaud et al., 2004; Palmela et al., 2018).The expansion of AIEC in the ileal mucosa has been implicated in the etiopathogenesis of Crohn's Disease (CD), a subset of IBD (Agus et al., 2014; J. G. Lee et al., 2019). We tested zinc treatment on NRG857c—a strain of AIEC isolated from the terminal ileum of patient with CD (Nash et al., 2010). NRG857c was shown to attach to intestinal epithelium and induce pro-inflammatory Th17 and Th1 responses in the murine intestine (C.-L. N. Small et al., 2013). We utilized the same pre-clinical mouse model to colonize mice with NRG857c. Mice used in this study were littermates and cohoused for two weeks following oral gavage with NRG857c. Mice were separated into two new cages and either given regular water (control) or zinc supplemented water (25mM ZnSO4-7HO) for 1 week (Figure 5A). We isolated fecal content from mice during the one week zinc treatment. After 3 days of zinc treatment (17dpi), we observed a significant reduction in fecal burden compared to untreated mice (Figure 5B). After 1 week of zinc treatment (21dpi), the average fecal burden of zinc treated mice dropped to 3.98E+03 CFU/g compared to 3.67E+05 CFU/g in the untreated mice (Figure 5B). Consistent with our fecal burden data, we also observed that the level of NRG857c in the

cecum (21dpi) was significantly reduced in the zinc-treated mice (group average=5.24E+03 CFU/g) compared to untreated mice (group average= 2.19E+05 CFU/g) (data not shown).

CD is characterized by inflammation that mainly affects the small intestine, but can be found anywhere along the GI tract (Ahmad et al., 2002). A major hallmark of CD involving the ileum (ICD) includes microbiota dysbiosis that favors increased colonization and expansion of pathobiont AIEC in the ileal mucosa (Baumgart et al., 2007). We found that NRG857c had successfully colonized the SI lumen (average=3.23E+04CFU/g) and ileal mucosa (average=2.85E+02 CFU/g) in our control mice (Figure 5C-D). We found that zinc treatment diminished NRG857c population in the SI lumen (average=1.81E+03 CFU/g) (Figure 5C). Ultimately, we could not recover AIEC from the ileal mucosa of zinc-treated mice (Figure 5D). This suggests that zinc treatment restricted growth of AIEC in the small intestine lumen and prevented NRG857c from inhabiting the ileal mucosa.

Manganese rescues antibacterial effect of zinc on AIEC *in vitro*

We next wanted to determine the effect of zinc supplementation on AIEC viability *in vitro.* To assess the direct antimicrobial activity of zinc on AIEC, we performed a bacterial killing assay. Briefly, we incubated 10^3 CFU AIEC with a range of ZnSO4-7H20 concentrations for 30 minutes at 37C. Percent killing was determined using controls as a baseline (average CFU from incubation in PBS alone). We observed that over half (75%) of AIEC were killed when exposed to 10uM zinc sulfate, indicating that LD50 is <10uM for AIEC. Exposure to concentrations of 25uM or higher result in ~90-100% killing of AIEC (Figure 6A).

To put these results into perspective, we next performed this killing assay with *E. coli* DH5a. We observed that at a concentration of 10uM, around 25% of DH5a were killed (as opposed to ~75% killing in AIEC group). For DH5a, the LD50 appears to be around 25uM, a concentration that killed 90% of AIEC, demonstrating an increased susceptibility of AIEC to excess zinc *in vitro* (Figure 6B). These results suggest that AIEC have enhanced sensitivity to the direct antimicrobial effects of zinc compared to a strain of non-pathogenic *E. coli*.

Genomic studies on AIEC show these microbes have acquired genes often found in enteropathogens that confer competitive fitness advantage, particularly within the inflamed intestine (Chassaing & Darfeuille-Michaud, 2013; Lucchini et al., 2021; Martinez-Medina et al., 2009; Nash et al., 2010; Sevrin et al., 2020; C. L. Small et al., 2016). This includes high-affinity ABC-type transport systems that are often utilized by pathogens to steal essential metals from their hosts(Nash et al., 2010). Interestingly, studies on manganese-specific ABC transporters (MnABC) in *S. pneumoniae,* demonstrate that these manganese transporters are highly susceptible to mismetallation under conditions of excess zinc (Couñago et al., 2014; Eijkelkamp et al., 2014; McDevitt et al., 2011). When exposed to high levels of environmental zinc, Zn(2+) ions bind to MnABC instead of Mn(2+), preventing uptake of Mn2+ and starving the microbe of essential manganese(Couñago et al., 2014) (Figure 6C). Many AIEC strains, including NRG857c, contain a MnABC transporter system(Nash et al., 2010). We next tested whether manganese supplementation could rescue AIEC from the antibacterial effects of excess zinc. Briefly, we incubated 10^3 CFU AIEC with added 100uM zinc sulfate, 100uM manganese chloride or 100uM zinc sulfate + 100uM manganese chloride for 30 minutes

at 37C. Based on our titration experiment, we chose to expose AIEC to 100uM zinc sulfate, as this concentration was fatal to AIEC (Figure 6A). Percent killing was determined using the average number of bacteria recovered from the control group (incubated with PBS) as a baseline. We found that 100uM zinc sulfate killed close to 100% of AIEC and the antibacterial effect of excess zinc was prevented by the addition of manganese chloride (Figure 6D). AIEC survival was unaffected by Mn supplementation alone. These results demonstrate that supplementation with manganese rescues antibacterial effect of excess zinc on AIEC. This further suggests that enhanced susceptibility of AIEC to excess zinc may be due to disruption of manganese uptake. Zinc-induced manganese starvation occurs due to mismetallation of transporters that are mainly found in pathogens and opportunistic pathobionts like AIEC. Therefore, zinc supplementation may be a strategy to target potentially harmful colonizers of the mucosal microbiota without disturbing commensal microbes.

Discussion

The mucosal microbiota is critical in shaping chronic intestinal inflammation (Atarashi et al., 2015; Baumgart et al., 2007; Leonardi et al., 2022; C.-L. N. Small et al., 2013). Colonization and expansion of adherent microbes in the mucosal microbiota exacerbate autoimmune conditions in susceptible individuals(Agus et al., 2014; Bradley et al., 2017; Flannigan & Denning, 2018; Y. K. Lee et al., 2011). In this study, we found that zinc treatment can selectively deplete adherent microbes from the mucosal microbiota. Zinc supplementation is known for its anti-inflammatory properties due to its direct effect on host immune responses (Chasapis et al., 2020; Foligné et al., 2020;

Kitabayashi et al., 2010). This is the first report demonstrating that zinc supplementation can also exert anti-inflammatory effects through remodeling of the gut microbiota.

Crohn's Disease is a chronic inflammatory bowel disease that often presents in young adults (20-30s) and most commonly affects the ileum (Ahmad et al., 2002; Baumgart et al., 2007). The overgrowth of AIEC in the mucosal microbiota has been implicated in both the etiology and progression of CD (Agus et al., 2014; Darfeuille-Michaud et al., 2004; Palmela et al., 2018; C.-L. N. Small et al., 2013). Patients with ICD (Crohn's Disease of the ileum) contain a selective increase of AIEC in their ileal mucosa (Baumgart et al., 2007). AIEC in mice were shown to adhere to IECs and upregulate pro-inflammatory T-helper cells including Th17 cells (C.-L. N. Small et al., 2013). In humans, AIEC expansion in the ileal mucosa is associated with increased inflammatory burden (Chervy et al., 2020; Glasser et al., 2001; Palmela et al., 2018). Here, we found that zinc supplementation reduces AIEC burden in the SI lumen and feces and prevents AIEC from retaining their niche in the ileal mucosa in mice. To date, there is no cure for CD, and available treatment options often include the use of antibiotics. However, excessive antibiotic use may be selecting for growth of pathobiont AIEC within the intestinal mucosa. NRG857c used in this study contain many genes responsible for resistance to antibiotics including tetracycline, chloramphenicol and beta-lactams (Baumgart et al., 2007; Nash et al., 2010). Moreover, AIEC may also be able to create their own biofilms, which could protect AIEC from antibiotic treatment(Chassaing & Darfeuille-Michaud, 2013; Martinez-Medina et al., 2009).

In addition to antibiotic resistance genes, AIEC have also acquired methods to evade host-imposed immunity. The host employs several immune strategies to ward off

microbes, such as AMP and mucus production(Okumura & Takeda, 2018). In the inflamed gut, the host secretes antimicrobial peptides that chelate metals, such as zinc, manganese and iron, to starve invading pathogens (Hood & Skaar, 2012; Raffatellu et al., 2009; Zackular et al., 2016). The bloom of AIEC in the intestinal mucosa in patients with CD is due to its ability to thrive in the inflamed gut (Kitamoto et al., 2020; McPhee et al., 2014; C. L. Small et al., 2016). AIEC contain genes involved in AMP resistance, enhanced acquisition of trace metals, regulation of flagella, and utilization of alternative carbon sources (Barnich et al., 2003; Kitamoto et al., 2020; Nash et al., 2010; Sevrin et al., 2020). Currently, there are no therapies to prevent growth of adherent microbes in the intestinal mucosa. Our work shows that AIEC strain, NRG857c, is sensitive to zinc supplementation *in vitro.* Intriguingly, we also see that manganese supplementation rescues antimicrobial effect of zinc on NRG857c. Zinc toxicity in bacteria is often due to dysregulation of metal ion homeostasis. Previous studies show that excess zinc can kill pathogens by starving them of essential manganese (Couñago et al., 2014; McDevitt et al., 2011). Our findings suggest that enhanced susceptibility of AIEC to excess zinc may be due to disruption of manganese uptake. Zinc-induced manganese starvation occurs due to mismetallation of manganese-specific ABC transporters (Couñago et al., 2014). These transporters are mainly found in pathogens to acquire manganese at high affinity and protect against oxidative stress (Eijkelkamp et al., 2014). Opportunistic pathobionts like AIEC likely acquired these transporters to overcome nutritional immunity and gain access to the intestinal mucosa. Therefore, zinc supplementation may be a strategy to target harmful colonizers of the mucosal microbiota without disturbing commensal microbes.

Our findings also show that zinc treatment exerts a powerful and fast-acting selection pressure against another adherent microbe, SFB, within the mucosal microbiota. The mechanism(s) underlying the profound sensitivity of SFB to zinc treatment remains an open area of investigation. Studies show that SFB likely co-evolved with their hosts to survive in direct contact with the intestinal epithelium(Atarashi et al., 2015). Genome sequencing of SFB strains show these microbes lack enzymes for the biosynthesis of critical amino acids and vitamins, indicating that SFB depend on the host for essential nutrients (Sczesnak et al., 2011). It is possible that zinc supplementation is disrupting bacterial and/or host-intrinsic responses that maintain homeostatic interactions between SFB and their hosts (Hedblom et al., 2018; Vaishnava et al., 2011). Consistent with this hypothesis, we found that zinc treatment enhanced antimicrobial activity of the ileal mucosa, which may prevent SFB from retaining their niche at the surface of intestinal epithelium. Future studies will have to be done to identify which host-intrinsic response(s) are involved in depletion of SFB in response to zinc treatment. Moreover, identification and validation of relevant SFB-intrinsic responses directly influenced by excess zinc continue to be hindered by lack of methods for independent culturing of SFB.

Methods:

Bacterial Strains

AIEC NRG857c (O83:H1) was isolated from an ileal tissue biopsy of a patient with CD (Berlin, Germany)(Nash et al., 2010). NRG857c was cultured in Luria Broth (LB) containing 100ug/ml ampicillin. For mouse experiments, NRG857c was grown overnight

and sub-cultured for ~4hrs prior to infection. *E.coli* DH5a (ThermoFisher) was grown in LB under sterile conditions.

Mouse handling

All animal studies were approved by the Institution Animal Care and Use Committees at Brown University. Age matched female C57BL/6 mice were used for all experiments and were ordered from Taconic Farms or Jackson Laboratory. Mice were maintained under a 12 hour light/ dark cycle and were monitored for signs of obvious physical

***In vivo* zinc supplementation**

Zinc supplementation experiments were conducted using age-matched WT C57Bl/6 mice from either Taconic Farms or Jackson Laboratory, as indicated. Mice were cohoused prior to start of each experiment. 25mM ZnSO4-7H20(Caisson Labs) was dissolved in 500mL drinking water and administered to mice ad libitum for 1 week. After 1 week, mice were anesthetized using isoflurane and then euthanized using cervical dislocation and tissues were harvested for downstream analysis.

Microbiome transfer into germ-free mice

Cecal content from 2 zinc supplemented mice and 2 control mice were collected. Samples were pooled per condition and gently spun down in 300 rpm in 6mL sterile 1X PBS for 5 minutes at 4C. Supernatant was collected and spun at 5,000rpm for 10 minutes at 4C. Pellet was re-suspended in 4mL 1x PBS+ 20% glycerol and kept on ice until oral gavage (200ul/mouse). Ex-GF mice were kept in a sterile hood for two weeks prior to sacrifice.

Pre-clinical model of AIEC colonization

Eight-week old female C57BL/6 mice were purchased from Jackson Labs and housed in a specific pathogen free (SPF) barrier facility under Level 2 conditions. AIEC colonization used in this study was based on the model shown in (C.-L. N. Small et al., 2013) with some modifications. Mice were given 20 mg streptomycin orally 24 hrs before infection and food was removed. Mice were infected with 2 x 10^9 CFU of AIEC NRG857c via oral gavage and were co-housed for two weeks. At 14dpi, mice were separated into two new cages and either given regular water (control) or zinc supplemented water (25mM ZnSO4-7HO) for 1 week. At 21dpi, mice were anesthetized using isoflurane and then euthanized using cervical dislocation. Tissues and fecal samples were weighed and homogenized in 1XPBS. For luminal burden, the ileum was flushed with 1xPBS to collect luminal content. The luminal content was spun down at 5,000rpm for 15 minutes and the dry pellet was weighed. Samples were serial diluted and plated on LB plates containing 200ug/ml ampicillin and kept at 37C overnight. Colonies were counted and AIEC burden was expressed as c.f.u/ g sample.

Small intestine lamina propria lymphocyte isolation

The distal half of the small intestine was flushed with 1X PBS and its Peyer's Patches were removed before being filleted and cut into 6-8 sections. Tissues were washed via vortexing for one minute in 1xPBS (twice) and dPBS (twice) for a total of 4 wash steps. Tissues were put in HBSS media containing 4.17mM NaHCO3+3% FBS + 1mM EDTA and shaken at 37C for 30 minutes at 200rpm. Tissues were then washed 4x as described

above and digested twice at 37C for 25 minutes in 12mL RPMI solution containing 0.025mg/ml collagenase (Sigma- Aldrich), 0.5mg/ml DNase1 (Sigma- Aldrich), and 0.25 units/ml Dispase (Sigma- Aldrich). Tissues must be vortexed for 1 minute after each digestion step to help remove cells. Cell suspensions were then strained through a 70 μm filter, spun down at 400xg for 10min at 4 degree, and separated on a discontinuous 40%/80% percoll (GE Healthcare) gradient. Cells at the interface were harvested and processed for flow cytometry analysis

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Flow cytometry analysis

As performed previously in our lab (Iyer et al., 2020), cells were stimulated for 4 hours at 37C in PRMI complete media containing 1X cell stimulation cocktail (eBioscience) and protein transport inhibitor cocktail (eBioscience). Cells were washed and stained for surface markers and viability for 30 minutes at RT. Samples were stained with viability dye eFluor 450 (ThermoFisher), CD45 evolve655 (ThermoFisher), CD4 BV785 (BioLegend), CD3 BV605 (ThermoFisher), CD8a PE-Texas Red (BioLegend), Following overnight fixation using the Fixation/Permeabilization solution (eBioscience Foxp3 Staining Buffer Set), cells were permeabilized and stained for cytokines IL-17A FITC (eBioscience), IL-22 PE (eBioscience), IL-10 PE-Cy7 (ThermoFisher) and IFNγ BV711 (eBioscience) and transcription factors RORgt PerCP-eFluor 710 (ThermoFisher) and FoxP3 (ThermoFisher). Cells were read on the BD FACSCelesta Cell Analyzer and data was analyzed using the FlowJo software.

16S Sequencing Analysis

PCR amplification was performed using the Phusion High Fidelity DNA polymerase with primers flanking the V4/V4 region of the 16S rRNA gene. These samples were submitted to the Genomics and Sequencing Center at the University of Rhode Island. Amplicons were sequenced on the Illumina MiSeq platform (2 x 300 bp paired end sequencing). The 16S rRNA gene sequences were processed using QIIME 2 v2019.4 pipeline(Bolyen et al., 2019), as done previously in our lab(Han et al., 2021).

RNA isolation and qPCR

Whole tissue RNA was extracted using PureLink RNA Mini Kit by Life Technologies and cDNA was synthesized using the M-MLV Reverse Transcriptase kit (Thermofisher). qPCR reactions were prepared using the Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific). Gene expression was normalized to GAPDH and relative expression values were determined using comparative ΔΔ Ct method. Primer information below:

gapdh FP	TGGCAAAGTGGAGATTGTTGCC
gapdh RP	AAGATGGTGATGGGCTTCCCG
il-17 FP	ATCAGGACGCGCAAACATGA
il-17 RP	TTGGACACGCTGAGCTTTGA
ifng FP	CACGGCACAGTCATTGAAAG
ifng RP	GTCACCATCCTTTTGCCAGT
il-10 FP	CCAAGCCTTATCGGAAATGA
il-10 RP	TTTTCACAGGGGAGAAATCG
saa-1 FP	CATTTGTTCACGAGGCTTTCC
saa-1 RP	GTTTTTCCAGTTAGCTTCCTTCATGT

saa-2 FP	TGTGTATCCCACAAGGTTTCAGA
saa-2 RP	TTATTACCCTCTCCTCCTCAAGCA
tnfa FP	TCGTAGCAAACCACCAAGTG
tnfa RP	AGATAGCAAATCGGCTGACG

Bacterial Load Measurement:

Fecal samples were collected, weighed and stored at -80C prior to DNA extraction. DNA was extracted using the Quick-DNA Fecal/Soil Microbe Kit by Zymo Research. Bacterial load was measured using quantitative real-time PCR (qPCR). qPCR reactions were prepared using the Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific) and the 16S rRNA gene 340F/514R primer pair (340F: 5'-ACTCCTACGGGAGGCAGCAGT-3', 514R: 5'- ATTACCGCGGCTGCTGGC-3'). Relative abundances of SFB was determined using SFB-specific primers (SFB_Forward: 5'-GACGCTGAGGCATGAGAGCA-3';
SFB_Reverse: 5'- GACGGCACGGATTGTTATTC-3') For relative abundance, SFB Ct values were normalized to the 16S rRNA gene Ct value and relative abundance values were determined using comparative ΔΔ Ct method.

***In vitro* killing assays:**

10^3 CFU AIEC NRG857c or DH5a was incubated with a range of ZnSO4-7H20 (Caisson Labs) concentrations for 30 minutes at 37C. Percent killing was determined using controls as a baseline (average CFU from incubation in PBS alone). For manganese rescue experiment, 10^3 CFU AIEC NRG857c was incubated with 100uM ZnSO4-7H20

(Caisson Labs), 100uM MnCl2-4H20 (Caisson Labs), or 100uM ZnSO4-7H20 + 100uM MnCl2-4H20 for 30 minutes at 37C. To assess antimicrobial activity of the ileal mucosa in response to zinc treatment, mucosal content was scraped from mice supplemented with 25mM ZnSO4-7H20 or control mice. 10^3 CFU of STM was incubated with mucosal content for 30 minutes at 37C. Percent killing was determined using the average number of bacteria recovered from the control group (incubated with PBS) as a baseline

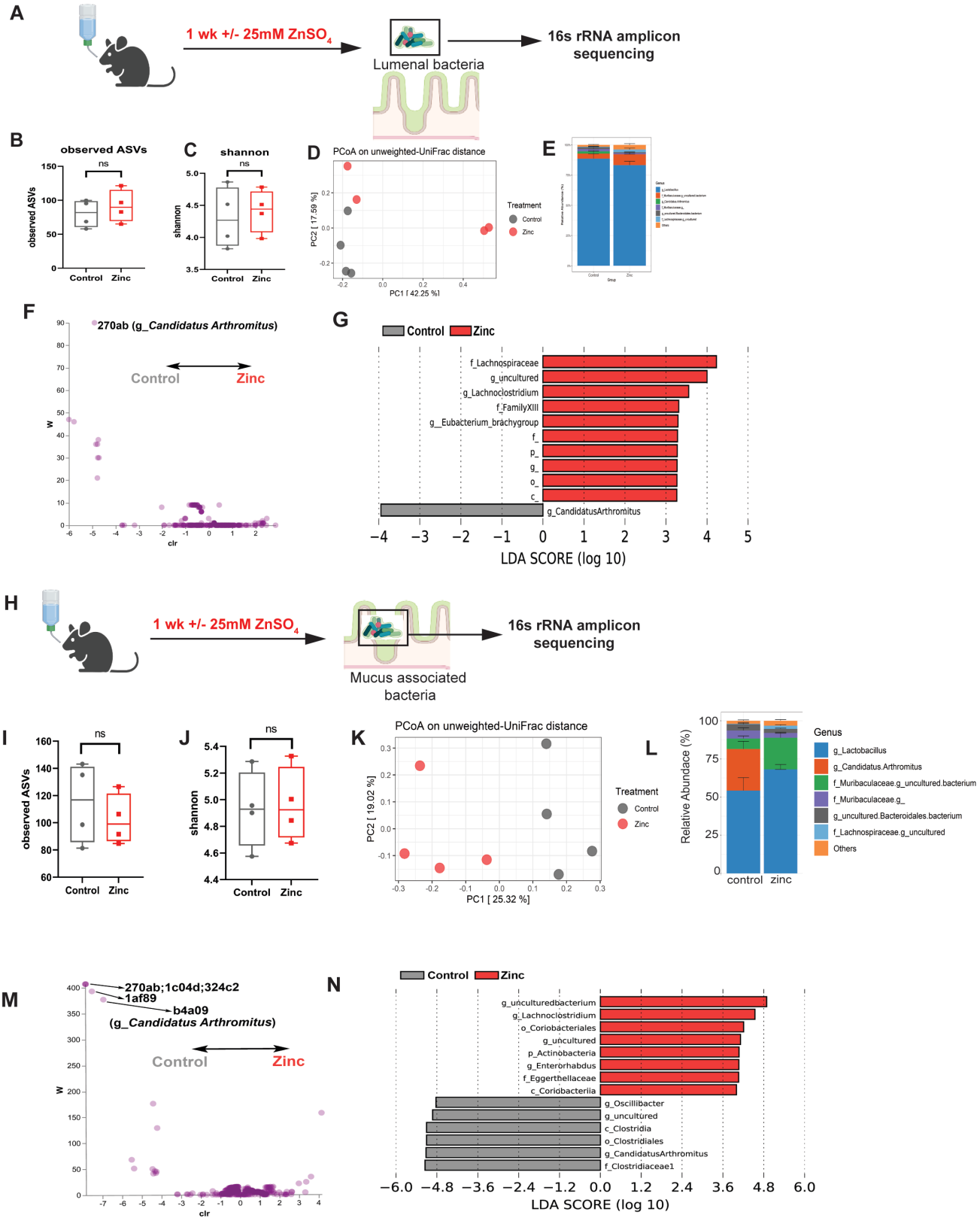

A
1 wk +/- 25mM ZnSO4
Lumenal bacteria
16s rRNA amplicon sequencing
B
observed ASVs
ns
observed ASVs
Control
Zinc
C
shannon
ns
shannon
Control
Zinc
D
PCoA on unweighted-UniFrac distance
PC2 [17.59 %]
PC1 [42.25 %]
Treatment
Control
Zinc
E
F
270ab (g_Candidatus Arthromitus)
Control
Zinc
W
clr
G
Control
Zinc
f_Lachnospiraceae
g_uncultured
g_Lachnoclostridium
f_FamilyXIII
g_Eubacterium_brachygroup
f_
p_
g_
o_
c_
g_CandidatusArthromitus
LDA SCORE (log 10)
H
1 wk +/- 25mM ZnSO4
Mucus associated bacteria
16s rRNA amplicon sequencing
I
observed ASVs
ns
Control
Zinc
J
shannon
ns
Control
Zinc
K
PCoA on unweighted-UniFrac distance
PC2 [19.02 %]
PC1 [25.32 %]
Treatment
Control
Zinc
L
Relative Abundace (%)
control
zinc
Genus
g_Lactobacillus
g_Candidatus.Arthromitus
f_Muribaculaceae.g_uncultured.bacterium
f_Muribaculaceae.g_
g_uncultured.Bacteroidales.bacterium
f_Lachnospiraceae.g_uncultured
Others
M
270ab;1c04d;324c2
1af89
b4a09
(g_Candidatus Arthromitus)
Control
Zinc
W
clr
N
Control
Zinc
g_unculturedbacterium
g_Lachnoclostridium
o_Coriobacteriales
g_uncultured
p_Actinobacteria
g_Enterorhabdus
f_Eggerthellaceae
c_Coriobacteriia
g_Oscillibacter
g_uncultured
c_Clostridia
o_Clostridiales
g_CandidatusArthromitus
f_Clostridiaceae1
LDA SCORE (log 10)

Figure 1: <u>Zinc treatment depletes adherent pathobiont, SFB, from the small intestinal microbiota</u> A) Experimental design: Taconic mice were supplemented with drinking water containing 25mM ZnSO4-7H20 for 1 week. Control mice received un-supplemented drinking water. Luminal content from the small intestine (SI) was harvested and analyzed by 16S rRNA amplicon sequencing. B-C) Alpha diversity (observed ASVs and Shannon Index) of SI luminal microbiota in zinc treated and control mice. D) PCoA plot based on unweighted UniFrac distance in the small intestine. E) Relative abundances at the genus level F) ANCOM generated volcano plot showing differential abundances of ASVs from zinc treated and control microbiotas. Positive clr indicates ASVs significantly enriched in zinc-treated samples, negative clr indicates ASV significantly enriched in control samples. Higher (w) indicates increased significance. ASVs classified as *Candidatus Arthromitus* are indicated by arrow. G) Histogram of the linear discriminant analysis (LDA) scores for differentially abundant bacterial clades in SI luminal samples between zinc-treated and control mice. Positive (red bars) LDA scores represent bacterial groups over-abundant in zinc-treated samples while negative (grey bars) represent bacterial groups overrepresented in control samples. H) Experimental design: Taconic mice were supplemented with drinking water containing 25mM ZnSO4-7H20 for 1 week. Control mice received un-supplemented drinking water. Mucosal scrapings from the ileum were harvested and analyzed by 16S rRNA amplicon sequencing I-J) Alpha diversity (observed ASVs and Shannon Index) of SI mucosal microbiota in zinc treated and control mice. K) PCoA plot based on unweighted UniFrac distance, *p=.03*. L) Relative abundances at the genus level from zinc-treated and control samples M) ANCOM generated volcano plot showing differential abundances between zinc treated and control microbiotas. ASVs classified as *Candidatus Arthromitus* are indicated by arrow. N) Histogram of the linear discriminant analysis (LDA) scores for differentially abundant bacterial clades in ileal mucosal samples between zinc-treated and control samples. Positive (red bars) LDA scores represent bacterial groups over-abundant in zinc-treated samples while negative (grey bars) represent bacterial groups overrepresented in control samples. Mann-Whitney test and PERMANOVA were used to assess significant differences in alpha and beta diversity N=4

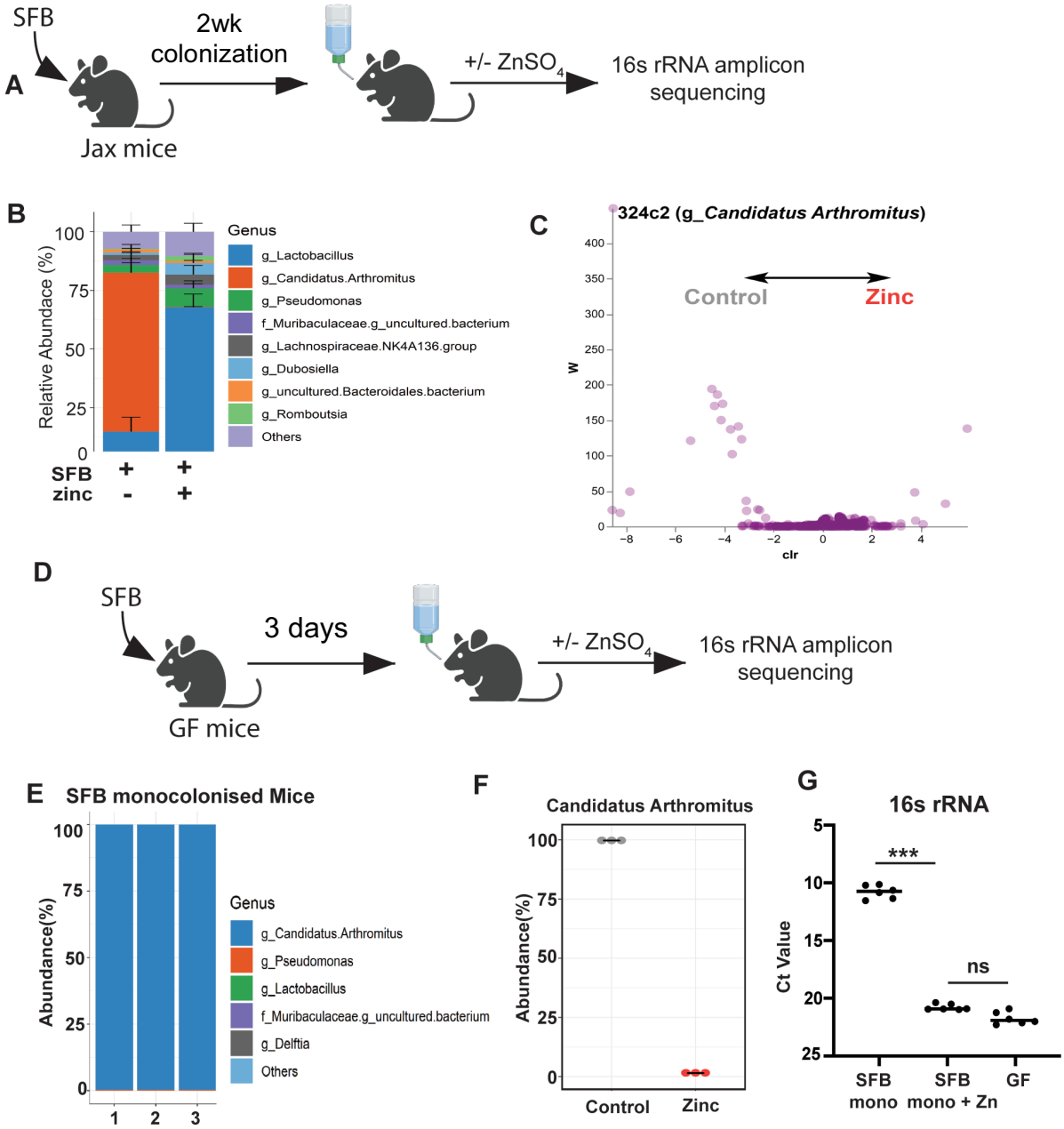

A
SFB
Jax mice
2wk
colonization
+/- ZnSO4
16s rRNA amplicon
sequencing
B
Relative Abundace (%)
100
75
50
25
0
SFB
zinc
+
-
+
+
Genus
g_Lactobacillus
g_Candidatus.Arthromitus
g_Pseudomonas
f_Muribaculaceae.g_uncultured.bacterium
g_Lachnospiraceae.NK4A136.group
g_Dubosiella
g_uncultured.Bacteroidales.bacterium
g_Romboutsia
Others
C
324c2 (g_Candidatus Arthromitus)
Control
Zinc
W
clr
D
SFB
GF mice
3 days
+/- ZnSO4
16s rRNA amplicon
sequencing
E
SFB monocolonised Mice
Abundance(%)
Genus
g_Candidatus.Arthromitus
g_Pseudomonas
g_Lactobacillus
f_Muribaculaceae.g_uncultured.bacterium
g_Delftia
Others
1
2
3
F
Candidatus Arthromitus
Abundance(%)
Control
Zinc
G
16s rRNA
Ct Value
***
ns
SFB
mono
SFB
mono + Zn
GF

Figure 2: **SFB colonization status is reversed by zinc treatment in conventional and germ free mice** A) Schematic showing experimental design. SFB was introduced into Jackson mice via oral gavage with fecal content from SFB mono-colonized mice. Mice were cohoused for two weeks and then randomly assigned into two groups (zinc treated mice received 25mM ZnSO4-7H20 in their drinking water for one week and control mice received un-supplemented water). Mucosal samples were collected and sent for 16S rRNA amplicon sequencing. B) Relative abundances at the genus level C) ANCOM generated volcano plot showing differential abundances between zinc treated and control microbiotas. ASVs classified as *Candidatus Arthromitus* are indicated by arrow. D) Schematic showing experimental design. SFB mono-colonized mice were supplemented with 25mM ZnSO4-7H20 in their drinking water, controls were given un-supplemented water. Mucosal samples were harvested and analyzed by 16s rRNA amplicon sequencing. E) Relative abundance data from SFB-monocolonized mice. F) Percent abundance of *Candidatus Arthromitus* from control and zinc-treated SFB mono-colonized mice. G) qPCR data showing 16s rRNA Ct value of fecal DNA isolated from SFB mono-colonized mice with and without zinc supplementation and compared to un-supplemented germ free mice. N=4

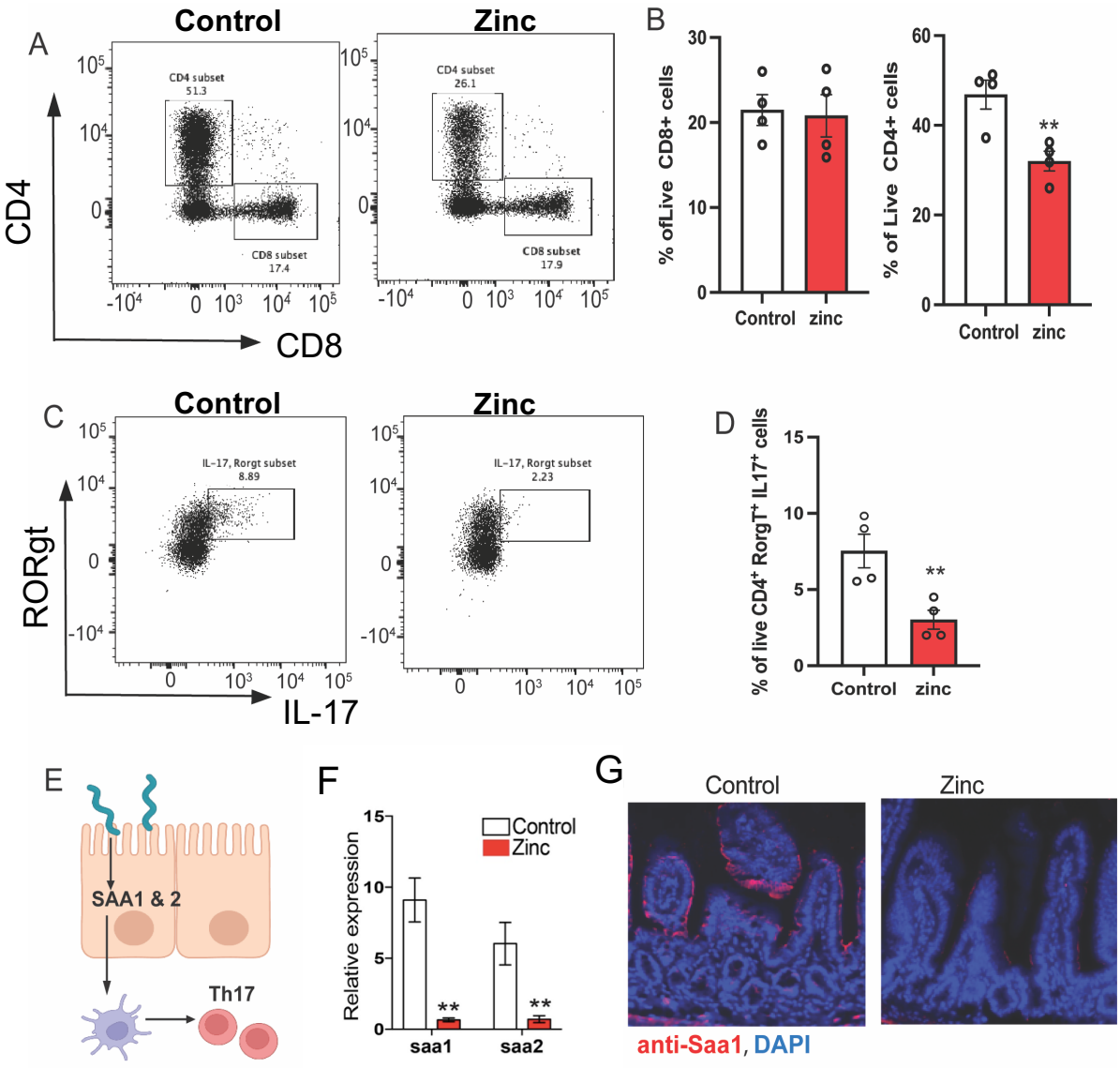


Figure 3: Zinc-treatment reduces intestinal Th17 cell population A-D) Small intestinal lamina propria lymphocytes collected from zinc treated mice and untreated (control) mice analyzed by flow cytometry. A-B) Gating for live CD4+ and CD8+ T-cell populations. C-D) Gating on live CD4+ T cells expressing RORgT and IL-17 for identification of Th17 cell population. E) Schematic showing upregulation of SAA1/2 in IECs in response to SFB adherence and the role this has on promoting Th17 cell differentiation. F) qPCR showing relative expression of *saa1* and *saa2* in the ileum of zinc treated and control mice. G) SAA 1 expression in small intestine sections from zinc treated and untreated mice via immunostaining with anti-SAA antibody. N=4.

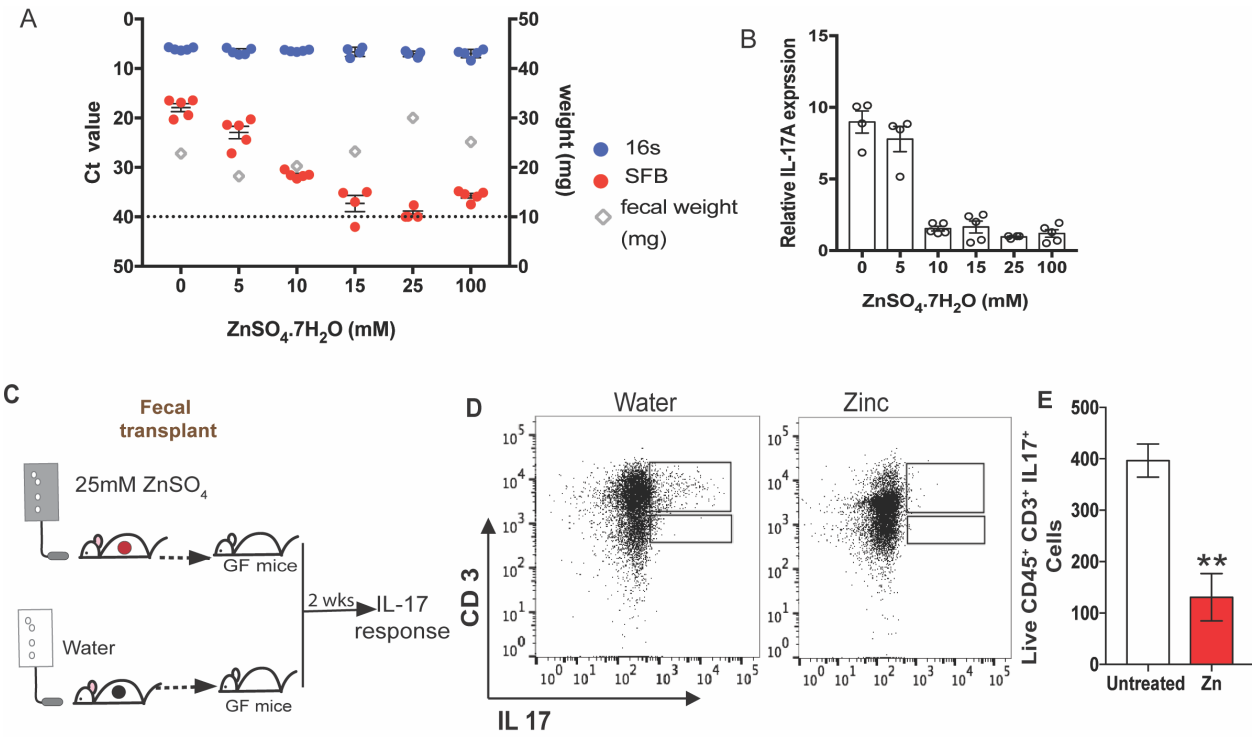


Figure 4: Zinc-treatment dampens Th17 cell induction through depletion of SFB
A-B) Taconic mice were supplemented with a range of zinc sulfate concentrations (5mM, 10mM, 15mM, 25mM and 100mM) for one week. A) Assessment of SFB and 16S DNA in feces using qPCR B) relative expression of *il-17* using qPCR. C) Fecal microbiome from Zn and control mice was engrafted in GF mice that were maintained on regular water and diet. D-E) After 2 weeks GF mice transplanted with fecal microbiome from Zn and control mice were analyzed for Th17 cell population (N=4-5 in each group).

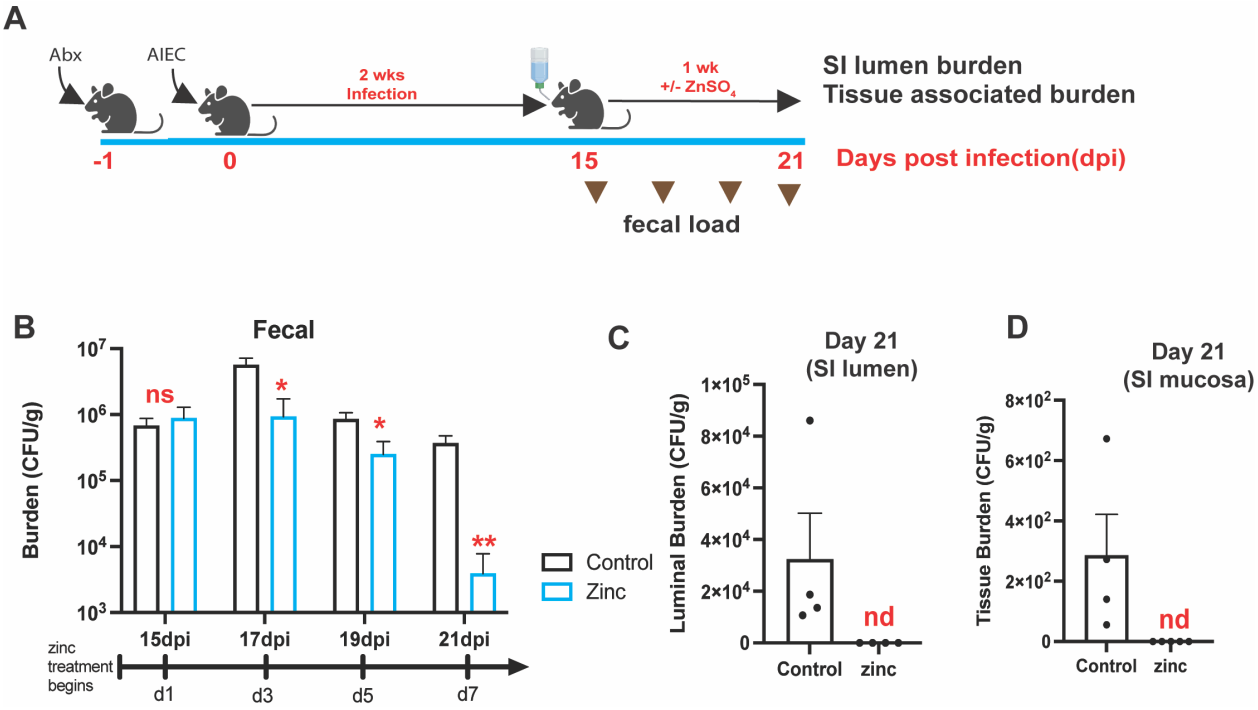


Figure 5: Zinc therapy reduces colonization burden and clears tissue adherence of human CD patient AIEC strain in a mouse model A) Mice were colonized with AIEC NRG857c as previously described (C.-L. N. Small et al., 2013). After two weeks, half of the mice were put on zinc supplemented water. Fecal burden of NRG857c strain was followed for 7 days, luminal and tissue adherent burden was quantified. B) Fecal burden of NRG857c strain in zinc supplemented and control mice C) Colonization level in the lumen of ileum of control and zinc treated mice after 1 week zinc treatment (21dpi) D) AIEC burden in the mucosa of the ileum of control and zinc treated mice after 1 week zinc treatment (21dpi). C-D) nd= not detected (N=4-5 in each group).

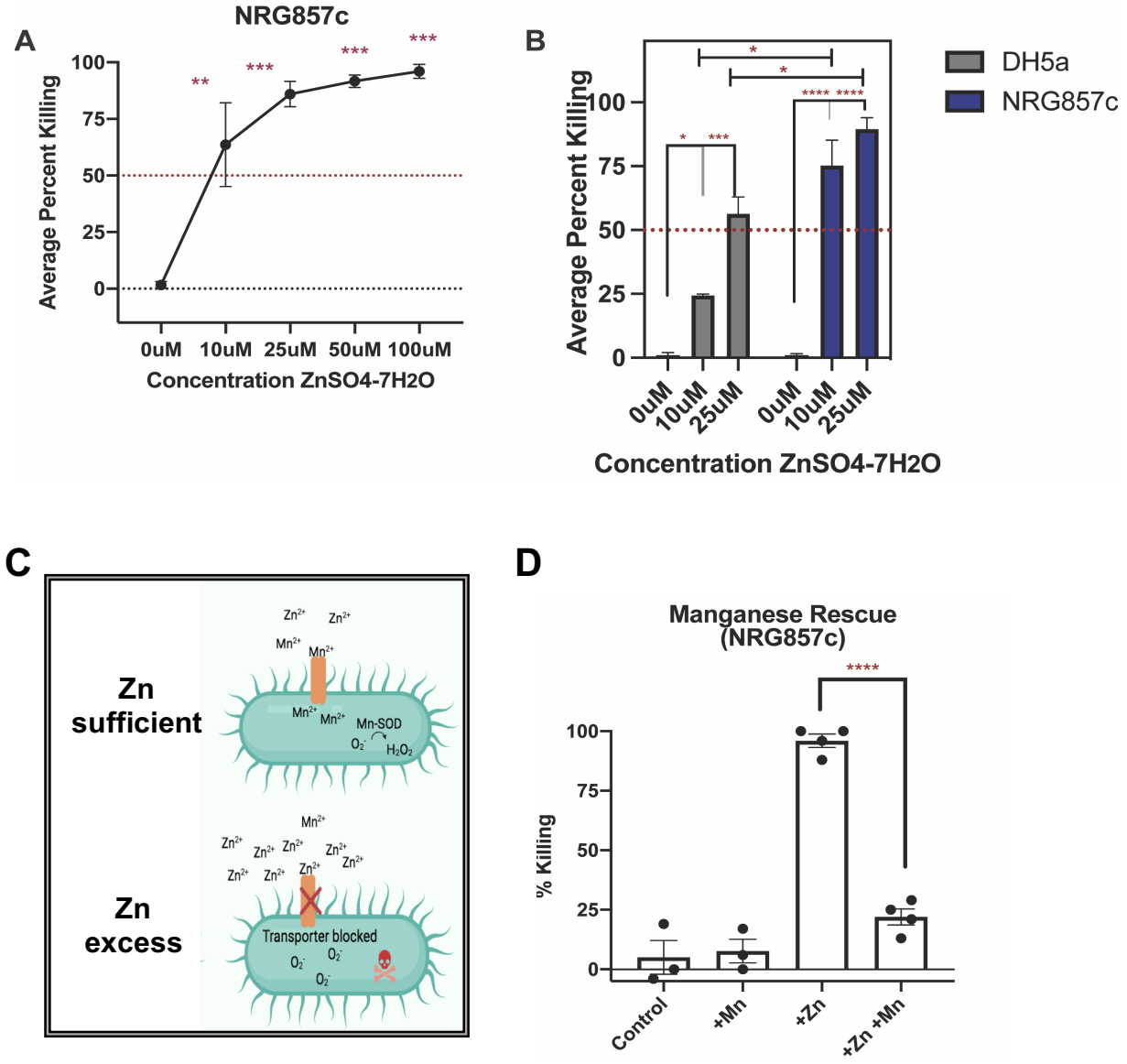
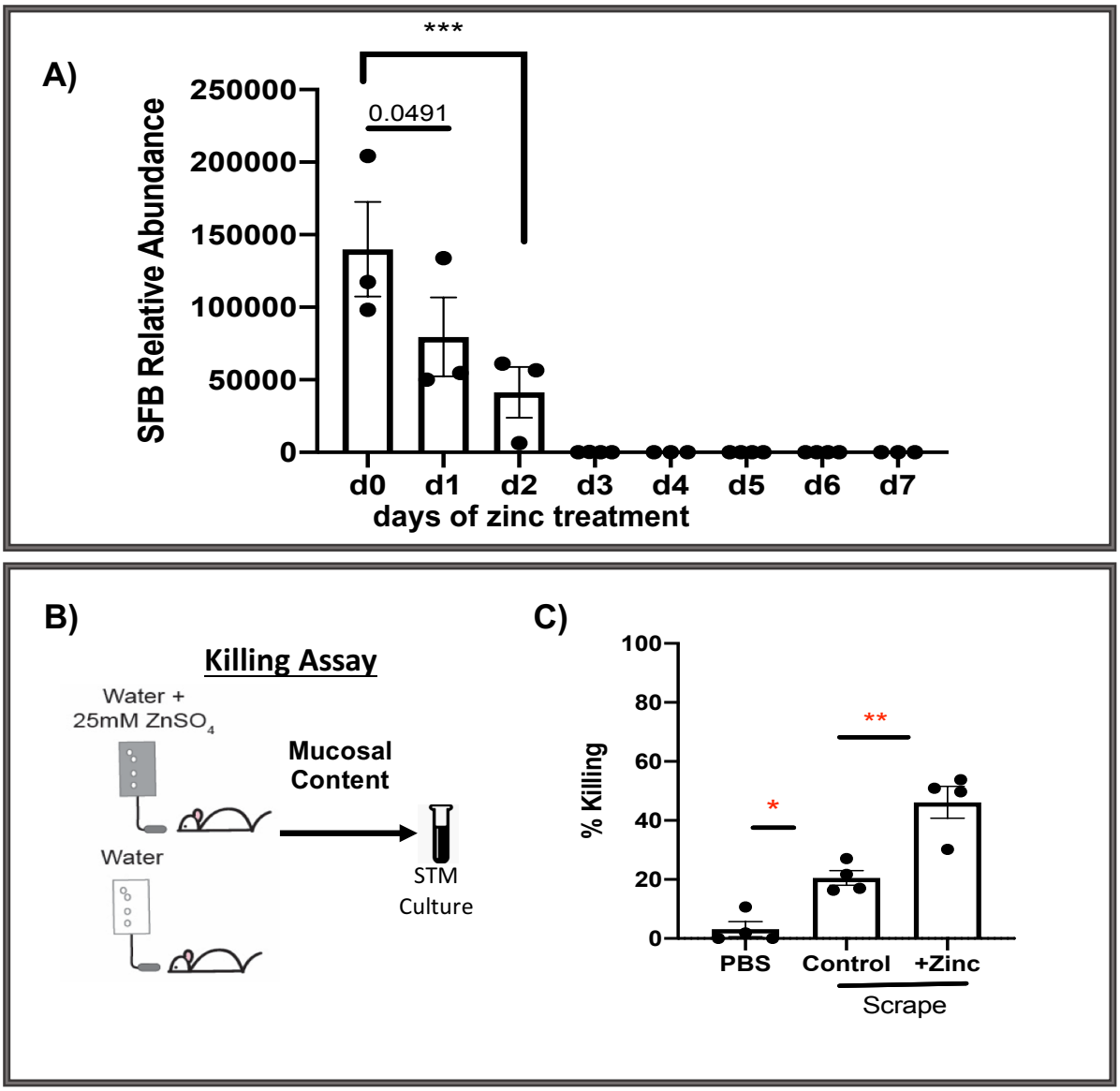


Figure 6: <u>Manganese rescues antibacterial effect of zinc on AIEC *in vitro*</u> A) AIEC NRG857c supplemented *in vitro* with different concentrations of ZnSO4-7H20 for 30 minutes. B) pathogenic (AIEC NRG857c) vs nonpathogenic (DH5a) *E. coli* strains compared for sensitivity to different concentrations of excess Zn C) Schematic showing pathogen susceptibility to excess Zn due to disruption of Mn uptake. Under excess zinc conditions, Zn2+ ions irreversibly bind manganese-specific ABC transporter (orange), which blocks Mn2+ uptake, thus starving these microbes of essential manganese and sensitizing them to oxidative stress. (Couñago et al., 2014; Eijkelkamp et al., 2014; McDevitt et al., 2011). D) AIEC NRG857c was exposed to excess Zn and/or Mn for 30 minutes *in vitro.*



Supplemental Figure 1: Short term zinc treatment (3 days) eliminates SFB and increases antibacterial activity of ileal mucosa A) Relative abundance of SFB in feces determined by qPCR. B-C) Antimicrobial activity of the ileal mucosa in response to zinc treatment, performed by a bacterial killing assay using mucosal (ileum) scrapings isolated from zinc-treated and untreated control mice. *Salmonella Typhimurium* (STM) was treated with mucosal content for 30min in 37C and plated. %Killing was determined using average CFU from incubation in PBS alone as a baseline.

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Chapter Three

The commensal microbiota regulates mobile zinc pool in intestinal epithelium through altered expression of Metallothionein 1 and Metallothionein 2

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The work in this thesis chapter was performed in the laboratory of Dr. Shipra Vaishnava, Ph.D. (SV). SG designed and conducted experiments, under the guidance of SV. SG and MW were involved in data collection. HL maintained and genotyped mice used in this study with help from SG. NM and SV designed MT1/MT2KO mice. Chapter was written by SG and edited by SV and SG.

Abstract:

Zinc is an essential micronutrient across the animal kingdom, making this metal highly sought after in the intestine. In humans and mice, zinc absorption is coordinated by a plethora of zinc transporters and zinc-binding proteins in enterocytes of the small intestine. Part of the cellular zinc machinery, Metallothionein 1 and Metallothionein 2 (MT1/MT2) are low molecular weight proteins that buffer and transport intracellular zinc. Here, we found that the commensal gut microbiota downregulates *mt1/2* in the intestinal epithelium. Using CRISPR/Cas9 MT1/MT2KO mice, we found these proteins are involved in restriction of mobile zinc in IECs. MT1/MT2 are stress-response proteins often upregulated during inflammation and infection, indicating a potential role of MT1/MT2 in sequestering zinc from pathogens and gut microbes that interact directly with IECs. This work provides foundational evidence of MT1/MT2 as microbiota-regulated, host-intrinsic factors involved in controlling mobile zinc at the host-microbe interface.

Introduction:

Zinc is an essential micronutrient across the animal kingdom, making this metal highly sought after in the intestine (Besold et al., 2017; Kehl-Fie & Skaar, 2010; Xia et al., 2021). The conserved importance of zinc is due to its function in protein stability, enzyme catalysis and cell signaling(Chasapis et al., 2012; Ohashi & Fukada, 2019). Zinc influences many cellular processes underlying antioxidant and immune responses (Kitabayashi et al., 2010; Siva et al., 2017; Sturniolo et al., 2001). Due to the necessity and toxicity of zinc, host and gut microbes must compete for zinc in the intestinal lumen,

as well as tightly regulate their intracellular zinc concentrations(McDevitt et al., 2011; Radin et al., 2019; Xia et al., 2021).

In the gut, a single layer of epithelial cells separates luminal microbes from underlying host tissue(Okumura & Takeda, 2018). In humans and mice, zinc absorption is mainly coordinated at the cellular level via enterocytes in the small intestine(Lee et al., 1989; Maares & Haase, 2020; Vagianos et al., 2007). Cellular zinc homeostasis is regulated by transporter families, ZIP and ZnT, and low molecular weight proteins, metallothionein 1 and 2. ZIP and ZnT control zinc influx or efflux, while MT1/MT2 bind zinc at low affinity to buffer levels of intracellular zinc ions (Costello et al., 2011; Coyle et al., 2002; Kimura & Kambe, 2016). Most cellular zinc is tightly bound to proteins and membranes(Bozym et al., 2006; Colvin et al., 2008; Li & Maret, 2009), making these ions less available for utilization by pathogens and gut microbes that directly interact with IECs. The coordination of zinc homeostasis in IEC, which involves constant acquisition and secretion of luminal zinc, changes the availability of this metal to gut microbes.

The host has evolved mechanisms, called nutritional immunity, which regulates the availability of nutrients to restrict or aid in microbial growth(Hood & Skaar, 2012). While most studies mainly focus on iron sequestration to limit bacterial growth in the gut, several studies indicate that zinc limitation and toxicity also contributes to nutritional immunity (Besold et al., 2017; Botella et al., 2011; Flo et al., 2004; Lopez et al., 2019; Raffatellu et al., 2009; Stocks et al., 2019, 2021). The discovery of calprotectin, a protein heterodimer secreted at mucosal sites that chelates zinc and manganese ions, demonstrates a role of zinc sequestration in nutritional immunity in the gut (Besold et al., 2017; Zackular & Skaar, 2018).

MT1/MT2 are stress-response proteins often upregulated during inflammation and infection, indicating a potential role of MT1/MT2 in nutritional immunity (Rahman & Karim, 2018). Here, we found that the commensal gut microbiota regulates mobile zinc levels in IECs through altered MT1/MT2 expression. Additionally, we found that MTs are highly expressed in cells located in the small intestinal crypts. Small intestinal crypts house highly specialized secretory epithelial cells, called Paneth cells, as well as the intestinal stem cell niche. These cells are sequestered to the base of the small intestinal crypt due to their critical roles in immunity, barrier integrity and tissue regeneration(Bel et al., 2017; Lueschow & McElroy, 2020; Vaishnava et al., 2008; Zhu et al., 2021). The role of zinc homeostasis in these specialized cell types of the intestinal epithelium remains understudied. This work provides foundational evidence of host-intrinsic factors involved in controlling mobile zinc at the host-microbe interface.

Results:

Gut Microbiota alters mobile zinc pool within IECs without disrupting total intestinal zinc levels

Numerous studies underscore the vital role of zinc in shaping mucosal immunity and maintaining gut integrity(Alkhouri et al., 2013; Ananthakrishnan et al., 2015; Chasapis et al., 2012, 2020; Kitabayashi et al., 2010; Ohashi & Fukada, 2019; Sturniolo et al., 2001). However, the effect of the gut microbiota on regulation of host zinc homeostasis is unknown. To begin investigating the contribution of the gut microbiota on intestinal zinc homeostasis, we assessed total zinc levels in small intestinal samples isolated from germ-free and conventional mice. Quantification of total zinc was determined using Inductively

Coupled Plasma Emission Absorbance Emission Spectroscopy (ICP-AES). We found that the total zinc in intestinal tissue was not significantly different between GF and CV mice (Figure 1D) maintained on similar diet, indicating that the presence of the gut microbiota does not change total intestinal zinc.

In addition to bound zinc, cells also contain a subset of mobile zinc (also referred to as labile or chelatable zinc), which are zinc ions that are not tightly bound to cellular structures/proteins. Mobile zinc pools accumulate in many specialized secretory cells, including Paneth Cells, and play important roles in cell function and signaling(Giblin et al., 2006; Podany et al., 2016). We next wanted to explore whether the gut microbiota regulates levels of mobile zinc at the host-microbe interface. To accomplish this, we compared levels of mobile zinc within IECs from GF and CV mice using flow cytometry with Zinpyr-1 probe. Zinpyr-1 (ZP1) is a membrane permeable, fluorescent sensor of mobile zinc (Chyan et al., 2014; Podany et al., 2016; Tomat et al., 2008). The Zinpyr-1 molecule, once bound to zinc ions, undergoes a conformational change that leads to a 3-5 fold increase in fluorescence intensity (Zhang et al., 2008). Intestines from CV and GF mice were harvested and IECs were obtained. Cells were then stained with EPCAM (epithelial cell marker), CD45, fixable viability dye (nonviable marker) and Zinpyr-1 (Figure 1A). Mean fluorescence intensity (MFI) of Zinpyr-1 stain was recorded for live epithelial cells (Live EPCAM+CD45-) (Figure 1B-C). We found that IECs from CV mice contained elevated ZP1 MFI compared to GF IECs (Figure 1C), indicating that the presence of the gut microbiota increases mobile zinc within intestinal epithelial cells.

Metallothionein levels in IECs are regulated by gut microbiota

Cellular zinc homeostasis is regulated by transporter families, ZIP and ZnT, and low molecular weight proteins, metallothionein 1 and 2 (Figure 2A). To identify host gene candidates underlying microbiota-induced changes in mobile zinc, we tested relative mRNA expression of 16 known cellular zinc homeostasis factors in the small intestine of GF and CV mice using qPCR. 8 of the 16 tested zinc homeostasis factors were differentially regulated between GF and CV intestines (*mt1, mt2, znt2, znt4, zip1, zip3, zip6, zip14)* (Figure 2B). We next wanted to determine whether these 8 factors were differentially regulated in IECs. To accomplish this, we utilized Laser Capture Microdissection (LCM) to isolate RNA from small intestine epithelial cells. Of the 8 gene candidates differentially regulated in whole tissue from CV and GF mice, only two were differentially regulated in IECs (*mt1 and mt2)* (Figure 2C). MT1/MT2 bind zinc at low affinity to buffer intracellular zinc. Our results demonstrate that the commensal gut microbiota differentially regulates many zinc homeostasis factors. However, with respect to IECs, the gut microbiota exclusively regulates *mt1/mt2* expression.

Increase in MT expression in antibiotic treated mice correlates with decrease free zinc in IECs

We next wanted to determine whether disruption of the commensal microbiota could alter mobile zinc status at the host-microbe interface. To accomplish this, we treated conventional mice with a cocktail of antibiotics (abx) for 1 week. Using flow cytometry, the mean fluorescence intensity (MFI) of Zinpyr1 stain was calculated in live epithelial (Epcam+CD45-) cells isolated from abx treated mice and control mice. We found that IECs from abx treated mice had a significant reduction in ZP1 MFI compared to controls

(Figure 3B), suggesting that disruption of the gut microbiota contributes to a loss of mobile zinc in the intestinal epithelium. Additionally, we saw that abx treated mice had a significant increase in intestinal *mt1* and *mt2* mRNA expression compared to controls, which suggests that abx treatment abrogates microbiota-induced suppression of MT1/MT2 (Figure 3A). Taken together, our results suggest a putative mechanism in which the commensal microbiota increases mobile zinc in the intestinal epithelium through suppression of MT1/MT2.

MT1MT2 control mobile zinc pool in IECs

Metallothioneins are highly conserved proteins across the animal kingdom (Coyle et al., 2002; Kimura & Kambe, 2016; Ruttkay-Nedecky et al., 2013). Mice contain two isoforms of metallothionein that are expressed in the intestine, MT1 and MT2, which have redundant function (Inoue et al., 2009). To further investigate the role of metallothionein in regulation of intestinal zinc homeostasis, we deleted mt1 and mt2 genes in mice using CRISPR/Cas9-mediated genome editing technology (MT1/MT2KO mice). Deletion of targeted region spanning most of the mt1 and mt2 gene was confirmed using PCR (Figure 4A). MT1/MT2KO mice do not express MT1 and MT2 proteins in the intestine (Figure 4C).

To gain further insight into the function of MT1/MT2 in the gut, we sought to identify its localization in the intestine. To accomplish this, ileal tissue sections from WT and MT1/MT2KO littermates were treated with an antibody that targets a conserved sequence in MT1 and MT2. We observed that MT1/MT2 staining was most pronounced within the crypts of Lieberkühn of the small intestine (Figure 4B-C), which house highly specialized,

secretory epithelial cells called Paneth cells. Interestingly, Paneth cells were shown to contain high levels of zinc, which accumulate in secretory granules along with antimicrobial peptides and other immune factors (Giblin et al., 2006; Podany et al., 2016). Contents from these secretory granules are released into the lumen, where they promote the spatial segregation of the gut microbiota and intestinal epithelium, defend against pathogens, and protect the intestinal stem cell niche (Bel et al., 2017; Vaishnava et al., 2008, 2011).

The intestinal stem cell niche is also sequestered at the bottom of the small intestinal crypts. These stem cells are responsible for the constant regeneration of the intestinal epithelium (Zhu et al., 2021). The pronounced MT1/MT2 expression at the base of the small intestinal crypt could also suggest an important role of MTs in regulating the intestinal stem cell niche. MTs are involved in cellular differentiation and proliferation, though the effect of MTs on these cellular processes changes based on cell type and disease state (Sankaramanivel et al., 2017). Preliminary data from our lab indicates that there is an increase in villi length in MT1/MT2KO mice compared to controls (Figure 4D), suggesting that MTs may be involved in regeneration of the intestinal epithelium. Intestinal cell turnover is important in maintenance of barrier integrity, response to pathogenic microbes, and nutrient absorption.

Another major function of the intestinal epithelium is to uptake nutrients from the diet. In mice and humans, epithelial cells in the small intestine are responsible for absorption of zinc and other trace metals from the diet (Lee et al., 1989; Maares & Haase, 2020). We next wanted to examine intestinal zinc uptake in MT1/MT2KO mice. To test this, we quantified levels of total zinc in the ileum of MT1/MT2KO and WT mice using

ICP-AES. We found that total intestinal zinc concentration was not different between MT1/MT2KO and WT mice (Figure 4E). [MOU1][MOU2]We also did not observe differences in intestinal Cu, Fe, Mn, and Mg concentrations between MT1/MT2KO and WT mice (Figure 4E). These results indicate that MT1/MT2 are not required for maintenance of total zinc and other trace metals in the gut.

We next wanted to assess changes in cellular zinc homeostats in MT1/MT2KO mice. Using flow cytometry, we assessed mobile zinc levels in IECs isolated from MT1/MT2KO and WT littermates. We found significantly elevated levels of mobile zinc in MT1/MT2KO IECs compared to WT IECs, as evidenced by increased ZP1 intensity in MT1/MT2KO cells (Figure 4F). Intestinal zinc is tightly coordinated at the cellular level, with many zinc homeostasis factors engaging in redundant or compensatory functions. It is possible that other zinc homeostasis factors are differentially expressed to compensate for loss of MT1 and MT2. We next evaluated the effect of MT1/MT2 deletion on expression of zinc homeostasis machinery in the intestinal epithelium using qPCR on LCM captured IECs. We found that zinc homeostasis factors were not differentially regulated between MT1/MT2KO vs WT IECs (Figure 4G). Taken together, these results suggest that MT1/MT2 are involved in regulating mobile zinc levels in IECs.

Discussion

Here we saw that MTs are highly expressed at the base of the small intestinal crypt. In addition to housing the intestinal stem cell niche, the small intestinal crypts also contain Paneth Cells (PCs). PCs were shown to accumulate large pools of mobile zinc in intracellular vesicles(Giblin et al., 2006). PC secretory vesicles contain antimicrobial

peptides, including Reg3g, alpha defensins and lysozyme, as well as zinc-dependent enzymes involved in antimicrobial processing, such as MMP7(Bel et al., 2017; Lueschow & McElroy, 2020; Vaishnava et al., 2008, 2011). Thus, mobile zinc pools in PCs can be secreted into the lumen or used for processing of antimicrobials. MT1/MT2 have been implicated in the control of subcellular zinc distribution. It is possible that MTs are involved in distributing mobile zinc pools within PCs, which could have impacts on PC antimicrobial function. Previously, ZnT2, an intracellular zinc transporter involved in zinc import into lysosomes, was shown to be important for secretion of antimicrobials in PCs (Podany et al., 2016), indicating a clear role of cellular zinc homeostasis machinery on PC antimicrobial function.

While engaging in nutritional immunity can be effective to prevent infection, host-imposed metal intoxication and starvation can also affect commensal microbes, especially those that exist closest to the intestinal epithelium. In chapter 2 of my thesis, we show that zinc supplementation alters composition of the gut microbiota by depleting adherent pathobionts. It is possible that MTs could be involved in shaping the composition of the mucosal microbiota through their role in distribution and sequestration of mobile zinc in IECs. Future studies using MT1/MT2KO mice should investigate the role of MTs in regulating adherent microbes, such as segmented filamentous bacteria or adherent invasive *E. coli*, in the intestinal mucosa.

Additionally, preliminary data from our lab shows that MT1/MT2KO mice have increased villi length in the ileum. This is intriguing, especially considering that MTs are highly expressed in the intestinal crypts, which contain the intestinal stem cell niche. This could suggest a role of MTs in differentiation of IECs. Alterations in villi height could

impact absorption of nutrients in the gut. In our study, we did not see changes in tissue zinc concentrations in the ileum of MT1/MT2KO vs. WT mice, which is consistent with previous work (Davis et al., 1998) . Epithelial cell renewal is also critical to maintain barrier integrity in response to damage. MT1/MT2 -null mice were previously shown to be better protected from DSS treatment, which causes epithelial cell injury, loss of epithelial cell barrier function, and severe intestinal inflammation, compared to WT mice(Devisscher et al., 2014). In our study, we saw that the commensal microbiota downregulates *mt1* and *mt2* expression, which may contribute to enhanced intestinal renewal and protection against epithelial cell injury.

MTs are also expressed in macrophages and neutrophils, where they play important roles in intracellular metal homeostasis and antioxidant responses in these cell types. (Dai et al., 2021; Inoue et al., 2009; Ruttkay-Nedecky et al., 2013; Subramanian Vignesh & Deepe, 2017). Previous studies report augmented humoral immune function, as well as changes in T and B lymphocyte proliferation and NF-kB activity, in MT1/MT2-null mice(Crowthers et al., 2000; Mita et al., 2002). A caveat of our study is that MT1/MT2KO mice are a whole-body knockout line. Deletion of MT1/MT2 in immune cells could also be affecting mucosal immune responses in the gut. Mucosal immunity is involved in regulation of homeostatic epithelial cell function, and can influence barrier integrity, Paneth cell function and intestinal regeneration. Additionally, the bidirectional relationship between the gut and other organs could contribute to phenotypes observed in MT1/MT2KO mice.

Methods:

Mouse handling:

All mice were bred in the SPF barrier facility at Brown University. MT1/MT2KO mice were generated using CRISPR/Cas9 technology at the Brown Transgenic Core. In mice, genes encoding MT1 and MT2 are located in close proximity on chromosome 8. Guide RNAs were designed to be complementary to sequences in exon 1 of *mt2* and exon 3 of *mt1*. *Stretpococcus pyrogenes* SF370 Cas9 protein was used to induce DSB at these target regions. This resulted in ~7400bp deletion spanning the majority of mt1 and mt2 genes. CRISPR/Cas9 MT1/MT2KO mice were be made in the C57BL/6 background. Littermates or co-housed mice were used to minimize microbiota-mediated effects on this study. Mice were maintained under a 12 hour light/ dark cycle and were monitored for signs of obvious physical stress during experimentation.

Germ-free C57BL/6 mice were bred and maintained in flexible film isolators at the gnotobiotic mouse facility run by our lab at Brown University. Air is supplied to each isolator by a blower attached to a filter by a flexible vinyl hose, allowing air to be sterilized before it enters the isolator. Supplies are autoclaved in stainless steel cylinders and are transferred into the isolator through a double door port. Diluted expor solution is used in both aerosolized and liquid form before items are brought into and out of the isolator. GF mice are monitored daily for any signs of distress. Sterile food and water are refilled once a week and sterile bedding is replaced every other week or sooner if needed. Sterility of each isolator is evaluated for viral and bacterial contamination using 1) culturing and 2) qpcr methods. For culturing, fecal samples are acquired from GF mice and are cultured

aerobically and anaerobically in three different medias. For qpcr, fecal samples are acquired from GF mice and DNA is isolated using the Quick-DNA Fecal/Soil Microbe Kit by Zymo Research kit. Bacterial contamination is assessed by amplification 16S rRNA gene as compared to previously confirmed GF and conventional DNA control samples.

ICP-AES

Small intestine samples were collected and flushed with dPBS. Tissues were tapped dry and weighed. Tissues were then digested in 70% nitric acid for 4 hours at 65^{o}C. Samples were diluted 1:25 in molecular grade water. Samples were run on the Thermo Scientific iCAP 7400 DUO ICP- Atomic Emission Spectrometer at the Environmental Chemistry Facilities at Brown University for metal quantification. Values were divided by tissue weight (ug/g).

Flow Cytometry

Epithelial cells were isolated from intestinal tissue via treatment of EDTA and by vortexing, which weakens epithelial cell adhesion. Stromal cells were then removed by filtration using a 70-micron strainer. Isolated IECs were then stained with EPCAM (epithelial cell marker), fixable viability dye (nonviable marker) and Zinpry-1 FITC (Cayman Chemical company). The Zinpyr-1 molecule, once bound to zinc ions, undergoes a conformational change that leads to a 3-5 fold increase in fluorescence intensity. The Zinpyr-1 probe has been shown to be zinc-specific and has an affinity for zinc too low to quench zinc ions already bound to proteins. Cells which were positive for

EPCAM and negative for viability dye were assessed for changes in mean fluorescence intensity of Zinpyr-1 staining.

Laser Capture Microdissection

Intestinal tissues were flushed with 1X PBS and OCT. Tissue sections were then embedded in OCT and stored at -80°C. Cryosections (10 µm thick) were stained with methyl green and eosin. Intestinal epithelial cells were selectively captured using the Arcturus Laser capture Microdissection system. RNA was isolated from IECs using the RNAqueous-Micro Total RNA kit (Ambion). RNA was converted into cDNA using the iScript cDNA synthesis kit (BioRad).

RNA isolation and qPCR

Whole tissue RNA was extracted from intestinal homogenate using PureLink RNA Mini Kit by Life Technologies and cDNA was synthesized using the M-MLV Reverse Transcriptase kit (Thermofisher). Quantitative real time PCR was carried out using SYBR green master mix. Gene expression was normalized to *gpadh* and relative expression values were determined using comparative $\Delta\Delta$ Ct method.

Microscopy

Tissues were fixed in formalin (1:10) and embedded in paraffin. Sections were deparaffinized and treated with Citrate Buffer pH 6.0 for antigen retrieval. Pirmary antibody used in this study targets a conserved region found in murine MT1 and MT2.

Anti- MT1/MT2 was raised in rabbit and created by Pacific Immunology Corp. Secondary antibody used was anti-Rabbit IgG Alexa 488 and all slides were stained with DAPI.

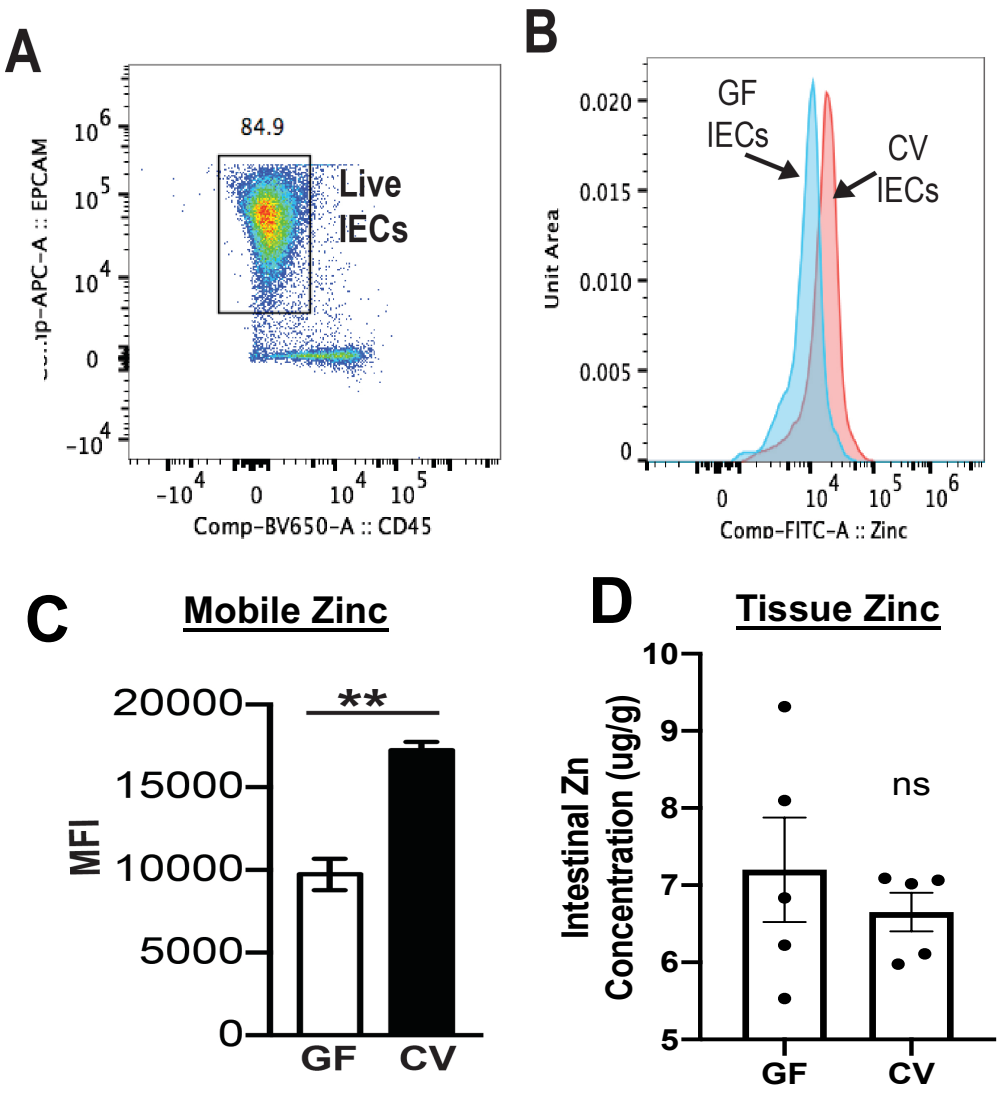

Figure 1: <u>Gut Microbiota alters mobile zinc pool within IECs without disrupting total intestinal zinc levels</u>. A) Live IECs were enriched from intestinal tissue of GF and CV mice by gating on live (Live Dead^{-}) intestinal epithelial (Epcam^{+}) cells. B,C) mobile Zn pool assessed in live CV and GF IECs using Zinpyr-1 (ZP1) probe. D) Quantification of intestinal zinc using ICP-AES

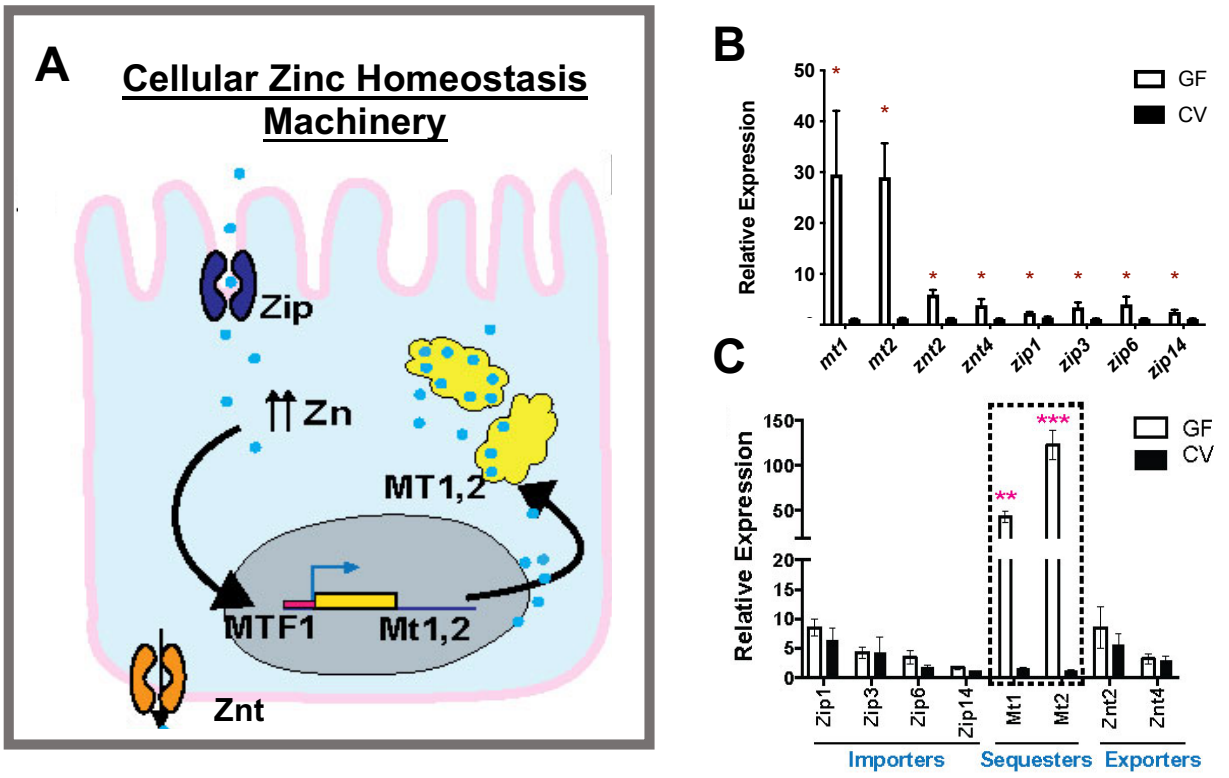

Figure 2: **Metallothionein levels in IECs are regulated by gut microbiota.** A) Schematic of Zn homeostasis machinery in IECs involving Zn transporters and binding proteins, B) qPCR analysis of Zn homeostasis factors differentially regulated in ileum from conventional (CV) vs. germ-free(GF) mice C) qPCR analysis of Zn homeostasis machinery in IECs from small intestine isolated via LCM. N=4, * p=0.05, **p=0.01, ***p=0.005, Students T test.

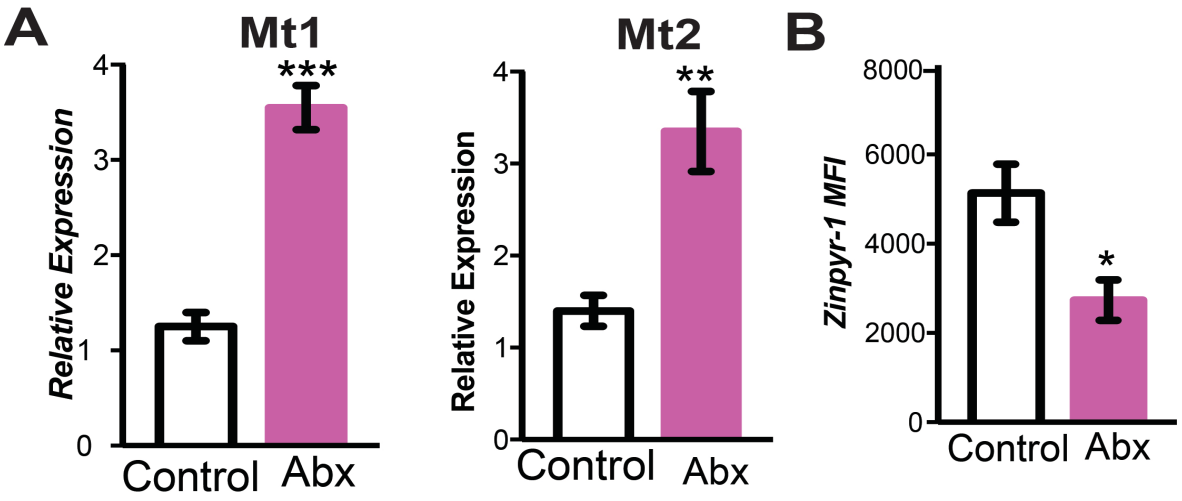

Figure 3: <u>Increase in MT expression in antibiotic treated mice correlates with decrease free zinc in IECs</u>. Mice were treated for cocktail of antibiotics for 1 week to deplete gut bacteria. A) Metallothionein expression in ileum of abx treated or untreated mice using qPCR B) To quantify free zinc levels in IECs mean fluorescence intensity (MFI) of Zinpyr1 was calculated in live Epcam+CD45- cells isolated from control mice and mice that were treated with antibiotic cocktail for 1 week. N=4, * p=0.05, **p=0.01, ***p=0.005, Students T test.

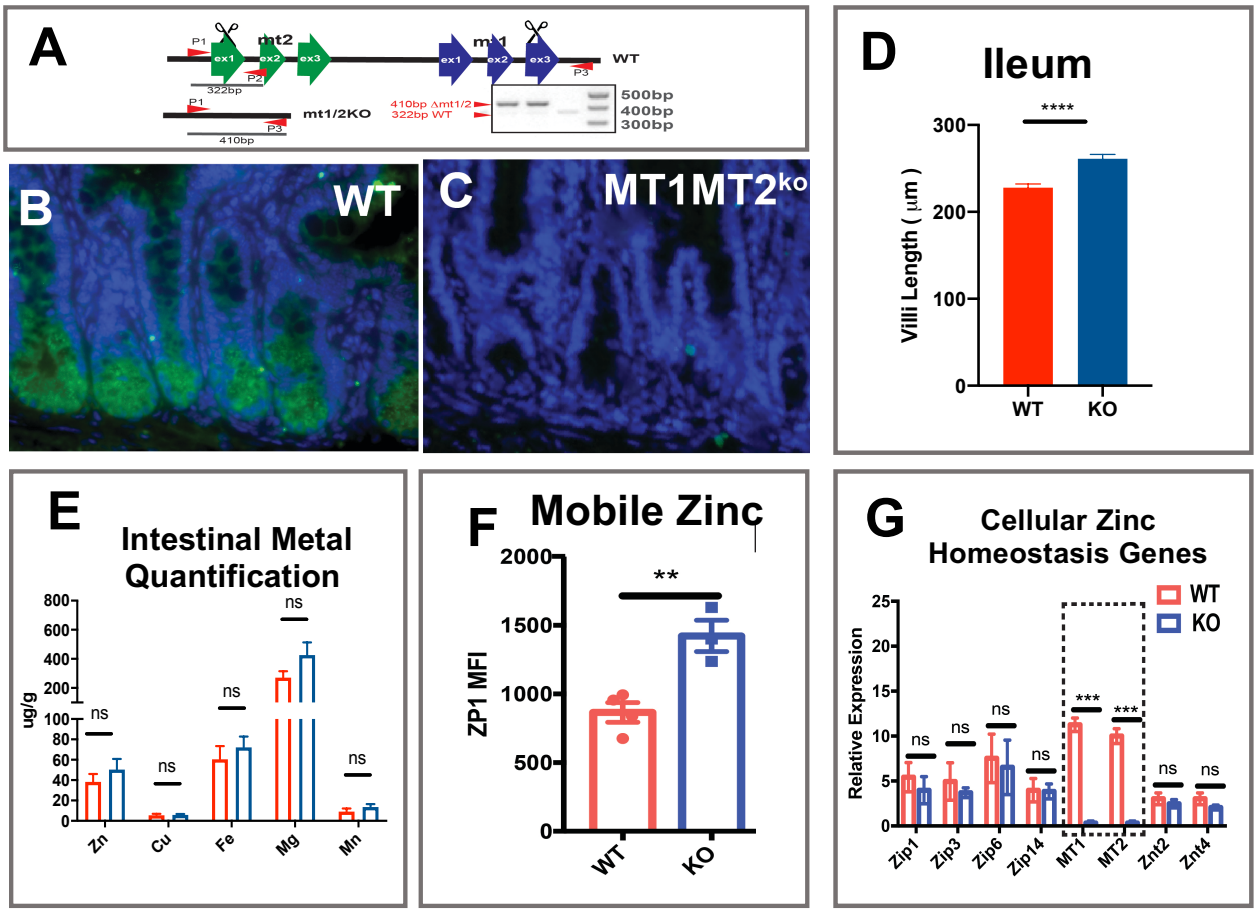

Figure 4. MT1MT2 control mobile zinc pool in IECs A) Strategy to create MT1MT2^{KO} mice using CRISPR technology and PCR analysis of mouse lines deleted for mt1mt2 locus. B,C) MT1MT2 staining in the small intestinal tissue of WT and KO littermates with anti-MT antibody (green). MT staining is most pronounced in small intestinal crypts D) Villi length in small intestine of WT and KO mice. E) Small Intestine tissue metal quantification using ICP-AES. F) mean fluorescence intensity (MFI) of ZP1 as a measure of mobile zinc levels in live Epcam+CD45- cells isolated from WT and KO mice G) Effect of MT1MT2 deletion on expression of zinc homeostasis machinery in IECs was evaluated by qPCR analysis of zinc transporters in LCM captured IECs N=4, * p=0.05, **p=0.01, ***p=0.005, Students T test.

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Chapter Four

Conclusion

Summary:

The importance of zinc on human health has been studied for over half a century. The anti-inflammatory properties of zinc are often attributed to its direct effect on immune cell differentiation and function(Chasapis et al., 2012, 2020; Kitabayashi et al., 2010a; Ohashi & Fukada, 2019; Xia et al., 2021). Chapter 2 of my thesis shows that zinc supplementation can alter the inflammatory potential of the gut microbiota by editing-out adherent pathobionts from the ileal mucosa. While adherent bacteria are found within the gut of healthy individuals, their expansion within the ileal mucosal microbiota is implicated in potentiating local and systemic inflammation underlying various autoimmune diseases, such as Inflammatory Bowel Disease, Rheumatoid Arthritis, and Multiple Sclerosis(Bradley et al., 2017; Darfeuille-Michaud et al., 2004; J. G. Lee et al., 2019; Y. K. Lee et al., 2011a; C.-L. N. Small et al., 2013). Here, we tested zinc supplementation on two adherent microbes, Segmented Filamentous Bacteria (SFB) and a strain of Adherent-Invasive E coli (AIEC) isolated from the ileal mucosa in a patient with Crohn's Disease. Zinc therapy selectively eliminated SFB from the murine mucosal microbiota, thereby dampening intestinal inflammatory T-cell induction *in vivo.* AIEC also displayed profound sensitivity to the antibacterial effects of zinc both *in vivo* and *in vitro.*

The host can regulate metal availability to restrict microbial growth in a process known as nutritional immunity. While most studies mainly focus on iron sequestration to limit bacterial growth in the gut, several studies indicate that zinc limitation and toxicity also contributes to nutritional immunity (Besold et al., 2017; Hood & Skaar, 2012; Kehl-Fie & Skaar, 2010; Lopez et al., 2019; Radin et al., 2019; Zackular & Skaar, 2018). The discovery of calprotectin, a protein heterodimer secreted at mucosal sites that chelates

zinc and manganese ions, demonstrates a role of zinc sequestration in nutritional immunity in the gut(Besold et al., 2017; Lopez et al., 2019; Zackular & Skaar, 2018). Metallothionein1/2 (*mt1/2*) were previously shown to be the most upregulated genes in the gut in response to zinc supplementation (Foligné et al., 2020a). Chapter 3 of my thesis demonstrates that MT1/2 are involved in regulating mobile zinc within the intestinal epithelium. Additionally, we found that MTs are highly expressed in cells located in the small intestinal crypts. Small intestinal crypts house highly specialized secretory epithelial cells, called Paneth cells. In addition to AMP secretion (Bel et al., 2017; Lueschow & McElroy, 2020a; Podany et al., 2016; Vaishnava et al., 2008, 2011a), PCs were also shown to accumulate large pools of zinc, which can be released into the intestinal lumen (Giblin et al., 2006). This work provides foundational evidence of MT1/2 as host-intrinsic factors involved in controlling zinc availability at the host-microbe interface.

Zinc supplementation dampens inflammatory potential of gut microbiota via depletion of adherent microbes

The mucosal microbiota is critical in shaping chronic intestinal inflammation(Duerkop et al., 2009; Flannigan & Denning, 2018; Hooper et al., 2012; J. G. Lee et al., 2019; Leonardi et al., 2022). Colonization and expansion of adherent pathobionts in the mucosal microbiota exacerbate autoimmune conditions including Crohn's Disease (Chervy et al., 2020; Flannigan & Denning, 2018). In my thesis work, I found that zinc treatment can selectively eliminate adherent microbes from the mucosal microbiota. Transfer of zinc-treated and untreated microbiomes into GF mice, demonstrate that loss of adherent microbe, SFB, in response to zinc supplementation

attenuates Th17 cell induction in the ileum. T-helper 17 (Th17) cells secrete pro-inflammatory cytokine IL-17 and are major contributors of autoimmunity throughout the body. Zinc treatment was also successful at clearing AIEC, an adherent pathobiont previously shown to trigger pro-inflammatory Th17 cell responses in the gut (Glasser et al., 2001; C.-L. N. Small et al., 2013), from the mucosal microbiota, as well as restricted its expansion throughout the intestine.

Zinc supplements are commonly prescribed to patients with CD, which can improve barrier integrity and reduce intestinal inflammation (Ananthakrishnan et al., 2015; Foligné et al., 2020a; Ohashi & Fukada, 2019; Sturniolo et al., 2001). The anti-inflammatory properties of zinc supplementation are often attributed to its direct effect on immune cell differentiation and function (Ananthakrishnan et al., 2015; Chasapis et al., 2012; Foligné et al., 2020b; Inoue et al., 2009; Prasad, 2014). It has been documented that *in vitro* zinc supplementation can suppress Th17 cell differentiation through altered STAT3 activity (Kitabayashi et al., 2010b). My thesis is the first report demonstrating that zinc supplementation can also dampen Th17 cell induction through remodeling of the gut microbiota.

In recent years, research has demonstrated that gut-derived Th17 cells can exacerbate extra-intestinal inflammatory diseases, such as Multiple Sclerosis and Rheumatoid Arthritis (Flannigan & Denning, 2018). Accumulation of Th17 cells in the gut can result in enhanced recruitment/ migration of Th17 cells to sites like the lung and central nervous system(Bradley et al., 2017; Y. K. Lee et al., 2011b). Therefore, the targeted depletion of adherent pathobionts in the gut in response to zinc supplementation may be involved in reducing inflammation throughout the body.

Current efforts to limit Th17 activity during autoimmune disease focus on the development of antibodies that neutralize IL-17 (Beringer et al., 2016). However, these efforts had adverse off-target effects in the gut (Hueber et al., 2012). Our work thus provides a novel nutritional approach for remodeling the microbiota to dampen inflammatory Th17 response in the gut.

Zinc supplementation as a therapy against Adherent-Invasive *E coli*

Crohn's Disease is a chronic inflammatory bowel disease that often presents in young adults (20-30s) and most commonly affects the ileum(J. G. Lee et al., 2019; Vester-Andersen et al., 2019). CD is likely caused by a combination of genetics and environmental factors, such as diet, microbiome, and prior intestinal infections(Alkhouri et al., 2013; Khan et al., 2020; C. L. Small et al., 2016; C.-L. N. Small et al., 2013; Vagianos et al., 2007). Major hallmarks of IBD include loss of intestinal barrier function and microbiota dysbiosis that favors a relative increase in *Proteobacteria(Palmela et al., 2018; Sartor, 2006; Sturniolo et al., 2001).* This includes a bloom of *adherent-invasive E coli (*AIEC*)* within the intestinal mucosa(Baumgart et al., 2007; Chervy et al., 2020; Glasser et al., 2001; J. G. Lee et al., 2019). AIEC are members of the human gut microbiota that can attach to and invade the intestinal epithelium(Glasser et al., 2001; Nash et al., 2010; C.-L. N. Small et al., 2013). In chapter 2, I found that zinc treatment cleared AIEC from the murine ileal mucosa in a pre-clinical model of AIEC colonization. Zinc supplementation also reduced AIEC burden in the SI lumen, cecum and feces.

Many patients with CD experience malabsorption of vital nutrients, including zinc, which results in patients becoming zinc deficient (Alkhouri et al., 2013; Siva et al., 2017; Vagianos et al., 2007). For this reason, zinc supplements are commonly prescribed to patients with CD, which can improve barrier integrity and dampen intestinal inflammation(Ananthakrishnan et al., 2015; Foligné et al., 2020a; Sturniolo et al., 2001). The protective effects of zinc supplementation are often attributed to the direct effect of zinc on host-intrinsic cellular processes (Foligné et al., 2020a; Ohashi & Fukada, 2019; Siva et al., 2017). In my thesis, I found that zinc supplementation can dampen inflammatory potential of the mucosal microbiota via depletion of adherent pathobionts, such as AIEC. This expands our understanding of the therapeutic function of zinc in treatment of CD to include its role in eliminating adherent pathobionts.

My thesis work indicates that zinc supplementation may be a strategy to selectively eliminate harmful colonizers of the mucosal microbiota without disturbing commensal microbes. Data from my thesis showed that adherent pathobionts displayed enhanced sensitivity to zinc supplementation compared to other members of the mucosal microbiota. Indeed, members of the commensal mucosal microbiota, such as *lactobacillus*, continued to thrive under zinc supplementation. The diversity of the small intestine luminal microbiota was also unaffected by zinc treatment. *In vitro* experiments showed that AIEC were more susceptible to zinc supplementation than non-pathogenic *E. coli,* further demonstrating a role of zinc supplementation in pathogenic microbes. Zinc supplementation is often recommended during advanced stages of CD to counteract malabsorption of zinc(Foligné et al., 2020a; Sturniolo et al., 2001). These data indicate

that zinc supplementation may be helpful in early treatment of CD to block overgrowth of harmful colonizers of the intestinal mucosa.

Importance of investigating microbiota at different regions within the intestine

Many studies characterizing the gut microbiome rely on feces for analysis. However, the gut contains distinct microbial populations that vary along the length of the intestine, as well as distance from the host epithelium (Baumgart et al., 2007; Leonardi et al., 2022; Vuik et al., 2019). These distinct microbial populations are shaped by environmental factors such as nutrient availability, host-imposed immune responses, pH and oxygen levels (Duerkop et al., 2009; Vaishnava et al., 2011b). Therefore, analysis of feces is not a complete representation of the gut microbiota.

Previous studies characterizing the effects of zinc excess on the gut microbiota focus exclusively on feces (Zackular et al., 2016), including one that reported limited changes in fecal microbiome structure (Foligné et al., 2020a). In my thesis work, I chose to explore the effect of zinc supplementation in shaping different microbial communities in the intestine (SI mucosal and luminal communities). By looking at distinct microbial populations other than feces, I discovered that zinc supplementation depletes adherent microbes within the mucosal microbiota. Many members of the ileal mucosal microbiota have potent immunomodulatory functions due to their proximity to host epithelium(Duerkop et al., 2009; Hooper et al., 2012; Ivanov et al., 2008, 2009; Leonardi

et al., 2022; Vuik et al., 2019). My thesis work underscores the importance of investigating different microbial communities within the gut.

Why are adherent pathobionts sensitive to zinc supplementation?

My thesis work shows that zinc treatment can selectively eliminate adherent microbes, SFB and AIEC, from inhabiting the ileal mucosa. Zinc supplementation is likely disrupting bacterial and/or host-intrinsic responses that confer persistence of adherent microbes at the host-microbe interface. The mechanism(s) underlying the profound sensitivity of adherent microbes to zinc treatment remains an open area of investigation.

Manganese rescues antibacterial effect of zinc on AIEC in vitro

In vitro experiments in chapter 2 of my thesis showed that AIEC were more susceptible to zinc supplementation than non-pathogenic E coli. This data suggests that the enhanced sensitivity of AIEC to zinc supplementation could be linked to genes that confer its pathogenic potential. However, genomic studies of AIEC could not identify a clear genetic basis for their pathogenic phenotype found *in vivo (Shaler et al., 2019).* Instead, AIEC appear to have acquired genes often found in enteropathogens that confer competitive fitness advantage, particularly within the inflamed intestine(Chassaing & Darfeuille-Michaud, 2013; J. G. Lee et al., 2019; Lucchini et al., 2021; Martinez-Medina et al., 2009; Nash et al., 2010; Sevrin et al., 2020; Shaler et al., 2019; C. L. Small et al., 2016). In addition to AMP resistance and flagella regulation, AIEC have also evolved ways to combat nutritional immunity posed by the host (Nash et al., 2010). This includes the use of high-affinity ABC-type transport systems to uptake and buffer cellular metal

concentrations(Nash et al., 2010; Radin et al., 2019). These transport systems contain a substrate binding protein (SBP) that scavenges either zinc, iron or manganese with high affinity.

Studies show that these ABC-type metal transport systems can be susceptible to mismetallation under conditions of zinc excess (Eijkelkamp et al., 2014; McDevitt et al., 2011; Radin et al., 2019). In the lung pathogen, *S. pneumoniae*, Zn2+ ions can competitively and irreversibly bind the SBP of the Mn- specific ABC transporter when zinc levels are high, thereby inhibiting manganese uptake (Eijkelkamp et al., 2014). This could indicate that gut bacteria harboring Mn-ABC transporters, such as AIEC, may be more susceptible to zinc toxicity, either induced by host immunity or through dietary supplementation. In chapter 2, I found that manganese supplementation rescues antimicrobial effect of zinc on NRG857c. These results suggest that zinc supplementation may selectively deplete AIEC via disruption of Mn2+ uptake. Future work will characterize the effect of zinc supplementation on intracellular Mn2+ or Zn2+ concentrations in NRG857c using ICP-MS

Manganese is an essential micronutrient that protects bacteria from oxidative stress. To combat host-imposed manganese sequestration and oxidative stress at the host-microbe interface, NRG857c and other members of the mucosal microbiota evolved various ways to acquire and/or store Mn2+. *Lactobacillus*, a main member of the mucosal microbiota, were shown to accumulate large amounts of inorganic manganese, which protects against oxidative damage by scavenging free radicals (Archibald & Fridovich, 1982; Gray & Jakob, 2015; Poddar et al., 2021). Meanwhile many strains of AIEC, including NRG857c, acquired high affinity Mn_ABC transporters, which can help them

uptake additional manganese, but may also make them susceptible to the antimicrobial effects of zinc supplementation (McDevitt et al., 2011; Nash et al., 2010). These different strategies in manganese homeostasis may underlie the profound sensitivity of AIEC to zinc treatment, while commensals within the mucosal microbiota, such as *Lactobacillus*, continue to thrive.

Studies show that mismetallation of Mn_ABC transporters, and subsequent manganese starvation, caused by excess zinc can sensitize pathogens to oxidative stress (McDevitt et al., 2011). The host-microbe interface is a toxic environment where bacteria must endure high levels of oxidative stress. This may explain the enhanced susceptibility of AIEC within the mucosal microbiota compared to other areas of the gut in response to zinc supplementation. Future work should investigate whether excess zinc heightens sensitivity to oxidative killing through manganese starvation.

Zinc supplementation enhanced the antimicrobial activity of ileal mucosa:

The host regulates the ecology and spatial segregation of the ileal mucosal microbiota through secretion of antimicrobial peptides (AMPs) (Lueschow & McElroy, 2020b; Vaishnava et al., 2008, 2011a). In response to SFB colonization, the host upregulates AMP production from IECs. AMPs such as Reg3g and alpha defensin 5 were shown to control growth of SFB(Farkas et al., 2015; Sano et al., 2015; Vaishnava et al., 2011a). In chapter 2, I found that zinc supplementation increases the antimicrobial activity of the ileal mucosa.

Zinc is important for processing and secretion of antimicrobial peptides from specialized IECs called Paneth cells(Giblin et al., 2006; Podany et al., 2016; Wilson et

al., 1999). Future studies should further investigate the effect of zinc supplementation on antimicrobial activity of the intestinal epithelium. This could include identifying expression levels of antimicrobials known to control levels of SFBs, such as Reg3g and alpha-defensins, in response to zinc treatment, using IHC.

However, these AMP responses likely would not impact AIEC colonization and expansion within the ileal mucosa. AIEC have acquired mechanisms that allow them to survive in the inflamed gut, including genes that confer protection against host AMP production (Shaler et al., 2019; C. L. Small et al., 2016). AIEC strain NRG857c (used in my thesis work) contains multiple genes that confer AMP resistance, including an outer membrane protease(Nash et al., 2010). Upregulation of host AMP production upon infection with enteropathogens drives AIEC expansion, thereby exacerbating disease severity (C. L. Small et al., 2016). The opportunistic bloom of AIEC in response to infection was dependent on bacterial genes that confer defensin resistance. Therefore, AIEC should not be sensitive to increased AMP activity of the ileal mucosa, and may actually benefit from it.

Potential role of nutritional immunity in controlling growth of adherent pathobionts?

To ward off invading microbes, the host can regulate nutrient availability to restrict microbial growth in a process known as nutritional immunity. Host-imposed zinc toxicity and starvation can help protect against infection(Besold et al., 2017; Hood & Skaar, 2012; Kehl-Fie & Skaar, 2010; Stocks et al., 2021). This includes calprotectin, which sequesters

manganese and zinc ions away from pathogens and nearby gut microbes (Lopez et al., 2019; Zackular & Skaar, 2018).

Adherent microbes are unique compared to other commensals due to their ability to bypass host immunity, including mucus and AMPs, to attach to the intestinal epithelium(Chervy et al., 2020; Martinez-Medina et al., 2009; Palmela et al., 2018; Sevrin et al., 2020; C. L. Small et al., 2016). AIEC contain genes involved in AMP resistance, enhanced acquisition of iron and other transition metals, regulation of flagella, and utilization of alternative carbon sources (Chassaing & Darfeuille-Michaud, 2013; Kitamoto et al., 2020; J. G. Lee et al., 2019; Nash et al., 2010; Shaler et al., 2019). These adaptations, among many others, allow AIEC to outcompete commensal microbes, gain access to the epithelial surface and thrive in the inflamed gut.

For these reasons, host-imposed zinc toxicity may be a necessary strategy to block colonization and expansion of adherent pathobionts, such as AIEC, in the intestinal mucosa. In the small intestine, specialized IECs called Paneth Cells (PCs) were shown to release large pools of mobile Zn(2+) into the lumen (Giblin et al., 2006). However, the molecular mechanisms underlying host-imposed zinc toxicity and the role this has on gut microbes is not known. In chapter 3 of my thesis, we identified metallothionein 1/2 as a potential host factor involved nutritional immunity at the epithelial barrier. In addition to controlling mobile zinc pools in IECs, we also saw that MTs are highly expressed in PCs. MTs may contribute to host immunity as 1) a host-secreted factor and/or 2) by changing levels of zinc secreted from PCs or 3) by activating defensins via zinc dependent metalloproteases.

Future work will further investigate the role of nutritional immunity in controlling expansion of adherent pathobionts at the host-microbe interface. Based on findings from my thesis, we generated two mouse lines: MT1/2KO mouse line (see ch 3) and epithelial-cell specific MTF-1 KO (Villlin-Cre MTF-1) mouse line. Metal-response element-binding transcription factor-1 (MTF-1) coordinates the expression of genes involved in zinc homeostasis, metal toxicity and oxidative stresses, including metallothionein 1 and 2. Future studies will characterize the mucosal microbiota and assess colonization and expansion of adherent pathobionts in these mice.

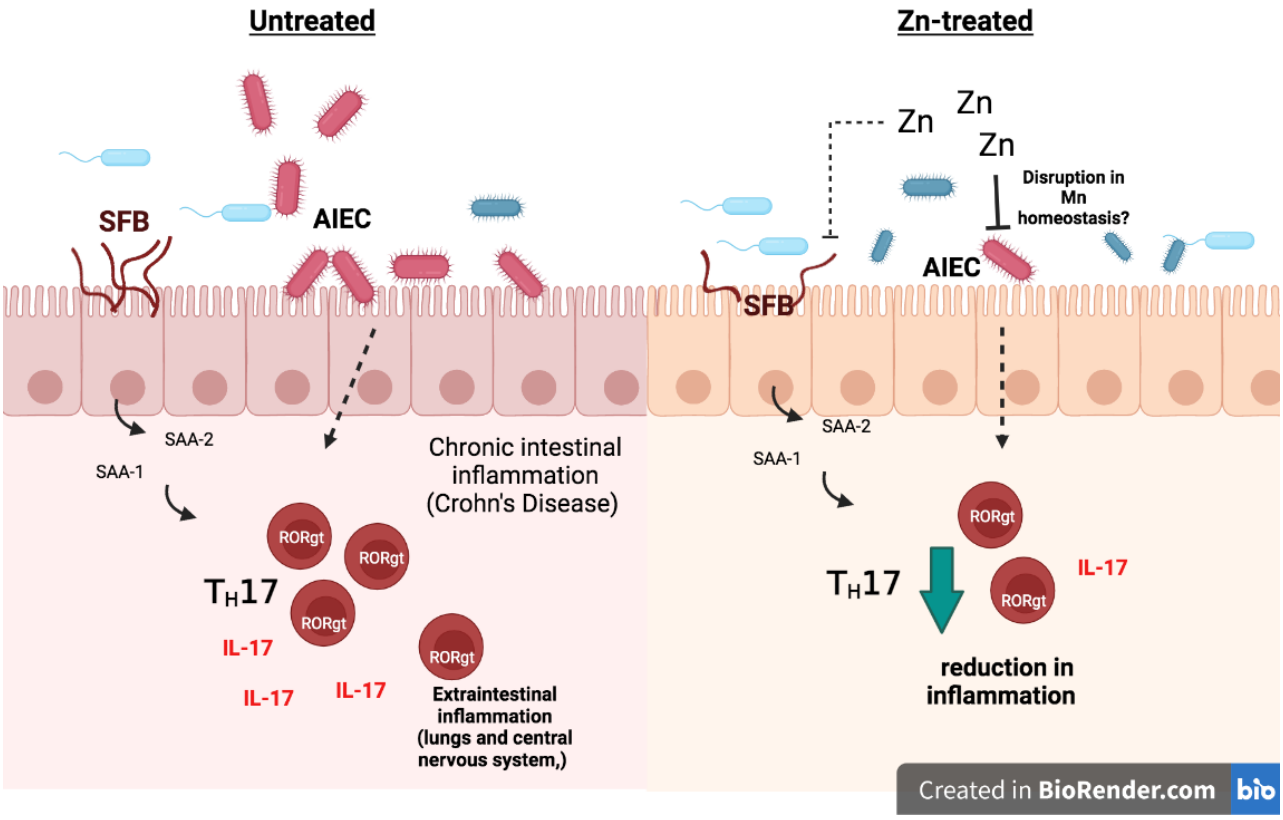

Figure 1: Zinc supplementation dampens intestinal inflammation and depletes adherent pathobionts from the mucosal microbiota. Adherent microbes attach to intestinal epithelial cells and induce Th17 cell differentiation in the gut. Th17 cells play important functions in a healthy gut, but can become autoreactive and contribute to inflammation throughout the body. In Crohn's Disease, the inflamed gut selects for bloom of adherent pathobiont, AIEC, in the ileal mucosa, which further potentiates inflammation. Currently, there is no therapy to prevent colonization and overgrowth of adherent pathobionts in the mucosal microbiota. Here, we found that zinc supplementation is effective at targeting adherent pathobionts—restricting their growth within the small intestine and preventing these microbes from retaining colonization of the ileal mucosal microbiota.

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