UNIVERSITY OF CALIFORNIA SAN DIEGO

Targeting siderophores to reduce adherent-invasive *E. coli* colonization: a potential therapy for Inflammatory Bowel Disease

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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The thesis of Suzana Hossain is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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LIST OF ABBREVIATIONS

AIEC	Adherent-invasive *Escherichia coli*
ANOVA	Analysis of variance
CD	Crohn's disease
CFU	Colony forming unit
CTB	Cholera toxin subunit B
DGE/Glc-Ent	Salmochelin
DMSO	Dimethyl sulfoxide
DSS	Dextran sulfate sodium
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
Ent	Enterobactin
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IgA	Immunoglobulin A
IL-10	Interleukin-10
Il10$^{-/-}$	Interleukin-10 deficient
LB	Luria-Bertani
LCN2	Lipocalin-2
Lcn2$^{-/-}$	Lipocalin-2 deficient
NHS	*N*-hydroxysuccinimide
OD	Optical density
PBS	Phosphate-buffered saline

p.i.	Post-infection
PP	Peyer’s patches
UC	Ulcerative colitis
WT	Wild type

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ACKNOWLEDGEMENTS

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Inflammatory Bowel Disease (IBD) is characterized by chronic intestinal inflammation that frequently results in alterations of the intestinal microbial composition and is a growing epidemic affecting millions of people worldwide. The current treatments for IBD mainly target the patients' immune system, but there is still no therapy that specifically targets the typical bloom of *Proteobacteria*, a major phylum of Gram-negative bacteria that includes various pathogenic species such as adherent-invasive *E. coli* (AIEC), a pathovar commonly associated with IBD. Bacteria require iron to survive in the host's iron-restricted environment, and certain pathogenic bacteria secrete small molecules known as siderophores to aid in iron acquisition. Strategies to impede iron uptake by pathogens, like siderophore immunization, may allow for a more targeted

strategy to reduce the *Proteobacteria* bloom during colitis. In this study, we investigated whether a vaccine that induces antibodies against the siderophore enterobactin (Ent) and the structurally similar salmochelin is protective against AIEC colonization in mice. We conjugated enterobactin to the immunogenic carrier Cholera Toxin B (CTB) and assessed the impact of CTB-Ent immunization on (I) the bloom of *Proteobacteria* and (II) providing protection against AIEC colonization during colitis. Immunized mice developed significant siderophore-specific antibody titers, and when the mice were infected with AIEC, they exhibited reduced AIEC colonization of the gastrointestinal tract. These results suggest that CTB-Ent immunization provided protection during infection with a siderophore-utilizing pathogen. Therefore, immunization against siderophores provides a more targeted approach in treating bacterial outgrowth of pathogens, which might be an attractive strategy to prevent AIEC outgrowth in patients with Inflammatory Bowel Disease in the future.

INTRODUCTION

Inflammatory bowel disease

Crohn's disease (CD) and ulcerative colitis (UC), the two main forms of Inflammatory bowel diseases (IBD), are characterized by chronic remitting and relapsing episodes of intestinal inflammation (Matsuoka & Kanai, 2014). In 2015, an estimate of 3.1 million U.S. adults suffered from IBD, which is a significant increase from the 1.8 million diagnosed in 1999 (Dalhamer et al., 2016). IBD results from complex interactions between the gut microbiota and the mucosal immune system on the basis of genetic susceptibility (Chang, 2020). It is speculated that the microbiota plays a crucial role in IBD as many patients exhibit microbial alterations and imbalances in the gut microbiota composition (Michail et al., 2011). Current IBD treatments primarily target the patient's immune system, but there is still no therapeutic strategy to specifically target and modulate the harmful players causing imbalance in the microbiota.

The human gut microbiota and mucosal immune response

The human gut hosts trillions of microbes constituting our microbiota (Clemente et al., 2012). The microbiota provides a wide range of benefits to our bodies including vitamin synthesis, development of immunity, and protection against invasive pathogens (Ducarmon et al., 2019). The healthy human gut microbiota consists primarily of protective commensal bacteria from the *Bacteroidetes* and *Firmicutes* phyla (Sekirov et al., 2010). *Proteobacteria*, the phylum containing commensal *Escherichia coli* but also pathogenic *E. coli* and certain human pathogens, are only present in small proportions in the healthy human gut microbiota (Rizzatti et al., 2017; Sekirov et al., 2010). Upon intestinal inflammation, however, *Bacteroidetes* and *Firmicutes* are frequently diminished while *Proteobacteria* including pathogenic *E. coli*, *Salmonella* spp., and *Shigella* spp., have a growth advantage and bloom (Zeng et al., 2017). Such alterations of the gut microbiota,

sometimes termed dysbiosis, and the pathological interactions with the gut immune system are prominent features of IBD (Michail et al., 2011). There is currently no therapeutic strategy to specifically modulate or impede this harmful bloom of *Proteobacteria* other than treatments to reduce intestinal inflammation.

In healthy individuals, a multi-component mucosal immune system keeps the intestinal microbiota in check. The gut barrier, a single layer of highly specialized intestinal epithelial cells, prevents direct exposure of bacteria to the underlying mucosal immune cells (Holmgren & Czerkinsky, 2005). If bacteria breach the epithelial barrier, the mucosal immune system orchestrates a series of events. During the mucosal immune response, innate immune cells like macrophages initiate an inflammatory immune response by releasing cytokines and other molecules to attract more immune cells including neutrophils (Holmgren & Czerkinsky, 2005). Macrophages are antigen-presenting cells that engulf and neutralize microbes, and present bacterial antigens to lymphocytic cells of the adaptive immune system (Arango Duque & Descoteaux, 2014). Upon antigen encounter, T lymphocytes release signaling molecules, known as cytokines, to regulate inflammation by inducing either pro-inflammatory or anti-inflammatory responses (Arango Duque & Descoteaux, 2014). Peyer's patches (PP), which are specialized areas of lymphoid follicles along the small intestine, harbor much of the B cells and T cells (Jung et al., 2010). These B and T lymphocytes induce antibody production in a highly orchestrated manner and thereby establish adaptive immunity (Cano & Lopera, 2013). PP are a considerable induction site of mucosal antibodies, particularly immunoglobulin A (IgA) (Jung et al., 2010). Once the affinity of antibodies in the PP has reached a maximum, IgA-secreting plasma cells migrate to the lamina propria, the effector site of the gut, and release commensal-specific antibodies into the lumen. Thus, IgA regulate the makeup and function of the microbiota and promote a symbiotic

composition of bacterial communities (Nakajima et al., 2018). However, this finely tuned mucosal immune system is dysregulated in IBD patients and may contribute to the pathogenesis of IBD (Xu et al., 2014).

Adherent-invasive *E. coli*

Although it is well known that *E. coli* is involved in the pathogenesis of IBD, a deep mechanistic understanding is still lacking. Previous studies have shown that IBD patients frequently have increased antibody titers against *E. coli* in comparison to healthy individuals (Darfeuille-Michaud et al., 1998). In particular, the pathovar adherent-invasive *E. coli* (AIEC), a functionally distinct and more virulent strain, is highly prevalent in patients with CD (Palmela et al., 2018). AIEC is able to colonize and adhere to intestinal epithelial cells and thereby disturbs the barrier between host tissue and commensal bacteria (Rolhion & Darfeuille-Michaud, 2007; Peterson & Artis, 2014). After adhesion, AIEC can cross the mucosal barrier, invade macrophages, continue replication, and constantly activate immune cells, leading to chronic inflammation (Hold et al., 2014; Glasser et al., 2001). Currently, there is no treatment strategy to specifically eradicate AIEC from IBD patients.

Iron acquisition via siderophores

Pathogens such as AIEC have to compete with the commensal microbiota for a multitude of nutrients to sustain their growth. Iron is an essential nutrient that almost all organisms require as it aids in biological processes like cellular respiration, DNA biosynthesis, and gene regulation (Andrews et al., 2003). Although iron is abundant in the environment, its abundance within organisms is tightly regulated. Iron mainly exists in its oxidized ferric state (Fe^{3+}), rendering it insoluble in physiological conditions (BaGG & Neilands, 1987; Kramer et al., 2019). Ferric iron

is also toxic in aerobic conditions as reactive oxygen species interact with Fe^{3+} in a way that is detrimental to living organisms (Andrews et al., 2003).

In order to circumvent these highly reactive and lethal conditions, pathogenic bacteria have developed strategies to acquire iron from the host's iron-restricted environment such as to synthesize and secrete small, iron-chelating molecules known as siderophores (Gerner et al., 2021). Siderophores have high specificity and affinity for ferric iron, so they can bind, or chelate, to Fe^{3+} to form a soluble complex that bacteria can then utilize for their own purpose (Andrews et al., 2003; Kramer et al., 2019). Some prominent siderophores of pathogens include enterobactin (Ent) and salmochelin (glucosylated form of Ent, Glc-Ent or DGE), which constitute important virulence factors for intestinal (enteric) pathogens (Gerner et al., 2021). Siderophores enable pathogens to scavenge iron and thereby starve out many members of the commensal microbiota. However, in response to invasive pathogens, Toll-like receptors on host immune cells orchestrate an innate immune response by transcribing, translating, and secreting lipocalin-2 (LCN2) (Flo et al., 2004). LCN2 then binds and sequesters enterobactin to ultimately prevent bacterial iron uptake. However, some pathogens have evolved to evade LCN2-mediated Ent sequestration, and produce additional siderophores that are not bound by LCN2. One of these siderophores is salmochelin (DGE), which is a glucosylated form of Ent. Strategies to impede the pathogen’s iron metabolism, such as targeting siderophores by mimicking the role of LCN2, might allow for a more specific treatment of AIEC colonization in IBD patients while preventing extensive perturbations of the commensal microbiota (as observed upon antibiotic administration).

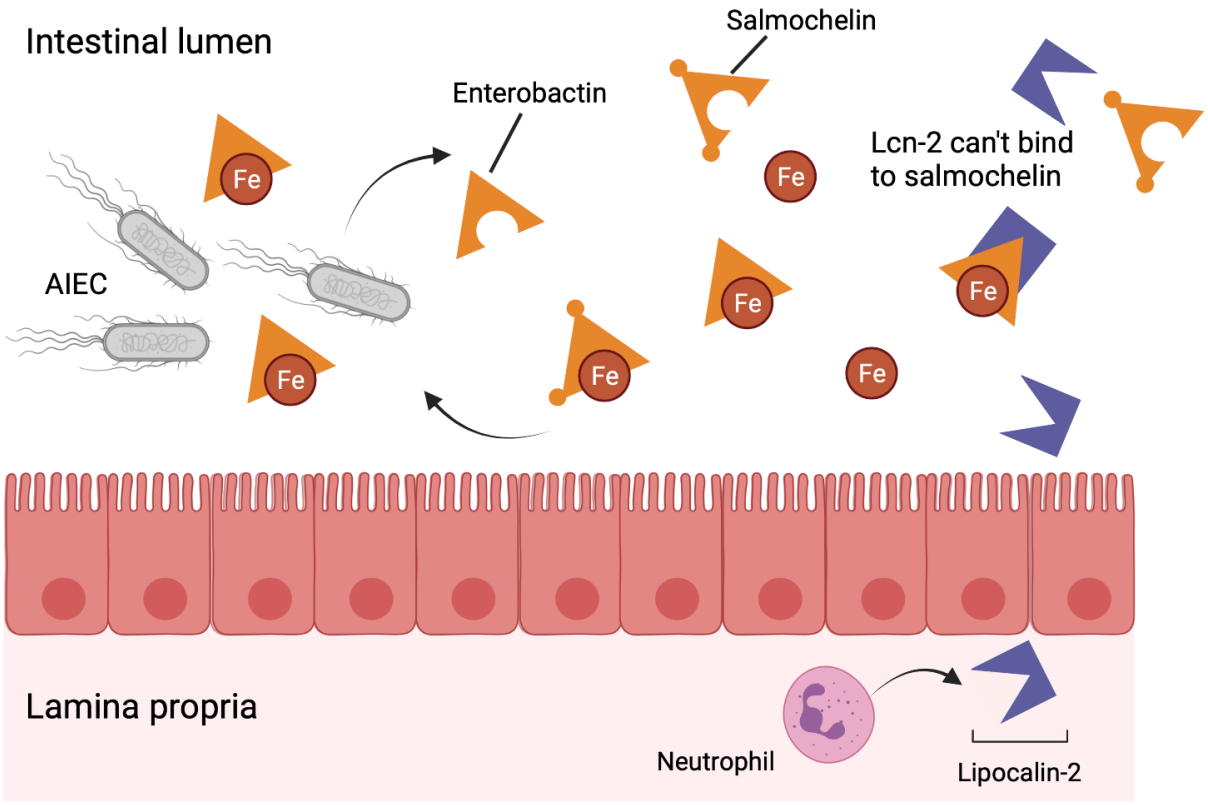


Figure 1. Mechanism of bacterial siderophore secretion upon invasion. During intestinal inflammation, the host's iron-restricted gut triggers AIEC to secrete the siderophores, enterobactin and salmochelin, that sequester iron, which is then utilized for AIEC's proliferation. In response, host epithelial cells and immune cells release lipocalin-2 that binds to enterobactin and thereby prevents bacterial iron uptake. Lipocalin-2 is unable to bind to salmochelin due to structural differences. Created with BioRender.com.

Siderophore immunization

Preliminary work from our lab found that immunization against siderophores in mice elicits a strong antibody response, thereby reducing the expansion of *Salmonella* spp. in the gut (Sassone-Corsi et al., 2016). In these studies, mice were intranasally immunized with a CTB-siderophore conjugate vaccine. Cholera toxin B subunit (CTB) is an immunogenic carrier protein that generates strong mucosal immune responses (George-Chandy et al., 2001). Studies conducted by George-Chandy et al. (2001) have shown that CTB conjugation lowered the antigen concentration required

to induce an immune response. Sassone-Corsi et al. (2016) proposed that once CTB is conjugated to the siderophore Ent, it should induce anti-Ent and anti-Glc-Ent antibodies in the immunized mice that would subsequently protect mice from *Salmonella* infection. To quantify these antibodies, the group established an in-house enzyme-linked immunosorbent assay (ELISA) and their results demonstrated that CTB-Ent immunized mice developed IgA antibodies that detected both Ent and Glc-Ent. These antibodies were found to be generated by B cells in the Peyer's patches (Sassone-Corsi et al., 2016). This first study focused on developing a more targeted approach to eradicate pathogens such as *Salmonella* from the gut while also highlighting a way to possibly limit its spread. For our study, we will adopt the method of siderophore-based immunization and assess its effects on various mouse models including *Lcn2*$^{-/-}$ mice, wild type mice, and *Il10*$^{-/-}$ mice, which is a genetic mouse model of IBD.

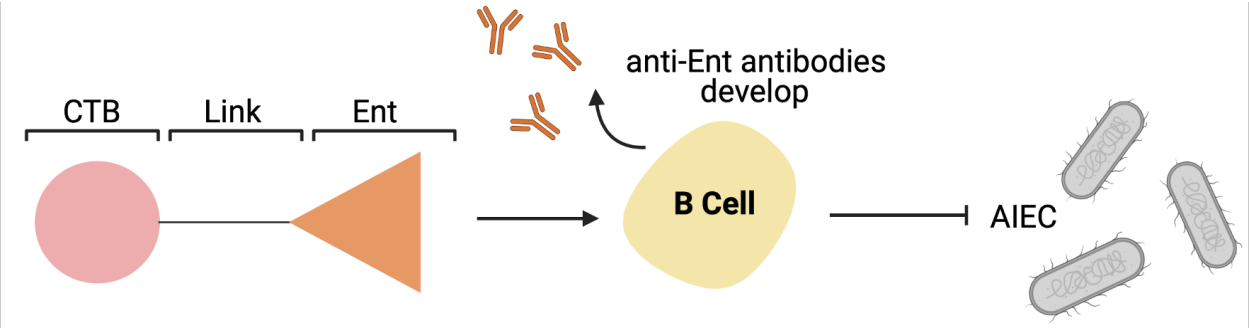


Figure 2. Siderophore-based immunization model. Siderophores, like enterobactin (Ent), can be made immunogenic by conjugation to a carrier protein like cholera toxin subunit B (CTB). Immunization with the CTB-Ent conjugate will initiate an immune response resulting in anti-siderophore antibodies that potentially provide protection against AIEC. Created with BioRender.com.

Il10$^{-/-}$ mice as a genetic model of IBD

Interleukin-10 deficient (*Il10*$^{-/-}$) mice are a genetically engineered model of IBD first generated by Kühn et al. in 1993 and have been widely used to study the etiology of IBD. These mice lack IL-10, an anti-inflammatory cytokine that inhibits proinflammatory responses and establishes immunologic tolerance (Arango Duque & Descoteaux, 2014). *Il10*$^{-/-}$ mice

spontaneously develop chronic intestinal inflammation (colitis), a signature of IBD (Kühn et al., 1993). However, the severity of intestinal inflammation is largely dependent on environmental factors such as the intestinal microbiota (Keubler et al., 2015). The absence of colitis in *Il10*$^{-/-}$ mice can be overcome by administering the nonsteroidal anti-inflammatory drug piroxicam, which results in an accelerated development of colitis (Hale et al., 2005). This genetic model of IBD along with our interest in siderophore immunization brings us to the aims of this study which are to assess the efficacy of CTB-Ent immunization in **(1)** blocking the bloom of *Proteobacteria* and **(2)** providing protection against AIEC colonization in the context of intestinal inflammation. Overall, this study could pave the way to the development of a more targeted IBD therapeutic.

METHODOLOGY

CTB-Ent conjugation

Ent-PEG_3-CO_2H was synthesized and obtained from Prof. Elizabeth Nolan's laboratory at MIT. The conjugation protocol for preparing CTB-Ent was adapted from *Siderophore-based immunization strategy to inhibit growth of enteric pathogens* (Sassone-Corsi et al., 2016). In order to activate the carboxylate group of Ent-PEG_3-CO_2H, solutions of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 100 μg/μL, Alfa Aeser) and *N*-hydroxysuccinimide (NHS, 60 μg/μL, Sigma) were prepared by dissolving to appropriate concentrations in DMSO. After solutions were dissolved to be completely clear, 12.5 μL of the EDC solution was added to the 2 mg aliquot of the Ent-PEG_3-CO_2H, immediately followed by the addition of 12.5 μL of the NHS solution. The reaction was incubated for 2h at room temperature with gentle rocking. While the reaction was incubating, the cholera toxin subunit B (CTB) solution was prepared.

CTB (2 mg, Sigma Aldrich) was dissolved in 500 μL of Milli-Q water. The solution was divided into two 250 μL aliquots, with one aliquot designated as the immunization control (CTB only). For removal of any residual azide and Tris buffer that are present in commercial CTB, both aliquots were added to separate, pre-wet Amicon ultra-0.5 centrifugal unit with an ultracel-10 membrane (3 kDa MWCO, EMD Millipore) and washed with 250 μL of sterile phosphate buffered saline (PBS, Gibco) by centrifugation 13,000 rpm x 10 min, 4°C). Subsequently the flow through was discarded and 300 μL of PBS was added to the filter and centrifuged for a total of 5 washes. After the fifth centrifugation, 400 μL of 0.1M sodium phosphate (NaP) pH 8.0 was added to the Amicon units and centrifuged. Flow through was discarded and the filter was washed 2 more times with NaP to buffer exchange the reaction buffer for a total of 3 washes. The final solution was

transferred to a new spin-filtration collection tube by flipping the column and centrifuging. The CTB solution was stored on ice until needed.

While the 2h incubation for the Ent-NHS/EDC reaction was near completion, 10 µL of iron (III) ethylacetoacetate ($Fe(acac)_3$, Sigma) was dissolved in DMSO to achieve a 212 mM concentration (37.4 mg/500 µL). 10 µL of 212 mM $Fe(acac)_3$ was added to the Ent reaction and incubated at room temperature for 10 min with gentle rocking. The colorless solution turned dark purple after addition of $Fe(acac)_3$, indicating that the iron-bound form was complete. Subsequently, the Ent solution was added to the CTB solution dropwise and incubated at room temperature for 3h with gentle rocking. After the incubation, 250 µL of PBS was added to dilute the crude reaction. The solution was divided into 2 aliquots (250 µL each) and transferred to 2 new spin-filtration units. 250 µL of PBS was added to each unit to top up the volume to a total of 500 µL. The units were centrifuged and the flow through was discarded. This was the first wash. 400 µL of PBS was added to adjust the volume back to 500 µL and centrifuged. This process was repeated 7 more times for a total of 8 washes to remove any coupling reagents. The concentration of CTB-Ent was determined by employing a Bradford protein assay and reading the plate at 595 nm. The concentration was adjusted to 2 mg/mL and the amount required in µL for animal immunization was calculated.

Mice and CTB/ CTB-Ent immunization

Wildtype (WT), *Lcn2*$^{-/-}$, and *IL10*$^{-/-}$ (all on a C57BL/6 background) mice were housed and bred in our vivarium. For immunization, 6-week-old male and female mice were briefly anesthetized with isoflurane and immunized by intranasal administration of either 100µg of CTB (control) or CTB-Ent (experimental). Mice were boosted 14 days after the first immunization with the same amount of CTB/CTB-Ent. Fecal samples were collected from mice prior to and after

immunization. Samples for 16S sequencing were snap-frozen in liquid N2. Additional fecal pellets were resuspended in PBS, homogenized and spun down at 10,000 x G for 10 min to obtain fecal supernatants. Samples were stored at -80 °C until further use.

AIEC strain and growth conditions

AIEC (strain NRG857c; isolated from the ileum of a CD patient; produces both Ent and Glu-Ent; gift from Prof. Brian Coombes, McMaster University) was grown in LB medium supplemented with chloramphenicol (30μg/ml) for 16–18 hours at 37°C with shaking. For mouse infections, AIEC was resuspended in PBS at the appropriate density and stored on ice until administration.

Colitis induction and AIEC infection of *Il10*$^{-/-}$ mice

Because *Il10*$^{-/-}$ mice do not develop spontaneous colitis under our housing conditions, intestinal inflammation was induced by piroxicam-supplemented chow (100 ppm; Teklad custom research diets, Envigo). Following complete immunization, mice were fed piroxicam-supplemented chow for a total of 10 days (food was changed daily). Animals were daily monitored for signs of disease and weighed every three days. On day 10 of piroxicam, mice were switched to regular mouse-chow for the remainder of the experiment and orally infected with 10^9 CFU of AIEC. Mouse weight and fecal shedding were monitored daily throughout the experiment, followed by serum and organ collection 120h post-infection.

Colitis induction and AIEC infection of WT and *Lcn2*$^{-/-}$ mice

WT and *Lcn*2$^{-/-}$ mice were immunized as described above and colitis induction and infection were done as follows. Briefly, 8-10-week-old mice were orally gavaged with 20mg of Streptomycin, followed by infection with 10^9 CFU of AIEC 24h thereafter. Mice were provided with 3% DSS (Alfa Aeser, MW 40,000) in the drinking water to induce inflammation and iron

restriction in the gut. Mouse weight and fecal shedding were monitored daily throughout the experiment, followed by serum and organ collection 120h post infection.

Fecal sample collection and preparation

Fresh fecal samples were collected in 1.7 mL Eppendorf tubes during several time points. The fecal pellets were weighed and resuspended in 1 mL of cold PBS. The samples were homogenized and used for fecal drop plating. After drop plating was complete, the samples were centrifuged (10,000 rpm x 5 min, 4°C) and the supernatants were collected and transferred to new tubes. The supernatants were stored at -80 °C until further needed.

Fecal drop plating

Homogenized fecal samples were diluted to a 6-log serial dilution (10^{-1} to 10^{-6}). 50 µL of the diluted samples were pipetted onto LB agar plates containing chloramphenicol using the drop plating technique. The spots were allowed to dry and were incubated at 37°C overnight. The next day, colonies from the best dilution (between 10 and 100 CFU) were counted and recorded.

ELISA

An ELISA assay was performed to measure anti-Ent and anti-DGE antibodies. Pierce Streptavidin High Binding Capacity Coated 96-Well Plates (Thermo Scientific) were washed 3 times with 200 µL of wash buffer (500 mL of PBS, 12.5 mL 1M Tris, 0.1% BSS, 0.05% Tween) per well. 100 µL of biotinylated enterobactin (Ent-Bio, 1µg/ml in wash buffer) or salmochelin (DGE-Bio, 1µg/ml in wash buffer) (both provided by Prof. Elizabeth Nolan, MIT) was added to each well and the plates were incubated for 2h at room temperature with gentle rocking. After incubation, the plates were washed 3 times with the wash buffer. Fecal supernatants were diluted in the wash buffer based on previous experiments. 100 µL of the diluted supernatants were added to each well and the plates were incubated for 1h at room temperature with gentle rocking.

Subsequently the plates were washed 3 times with the wash buffer, followed by the addition of IgA-HRP or Ig-HRP antibody (1:1,000 dilution in wash buffer). 100 µL of the antibody dilution was added to each well and the plates were incubated for 30 min at room temperature with gentle rocking. During incubation, the substrate solution was prepared by dissolving one *o*-phenylenediamine dihydrochloride (OPD, Sigma) tablet and one urea hydrogen peroxide tablet (Sigma) into 20 mL of UltraPure distilled water (Invitrogen). After incubation, the plates were washed 3 times with the wash buffer and 100 µL of the substrate solution was added to each well and incubated for 20 minutes in the dark. The reaction was quenched by adding 50 µL of 2N hydrogen sulfate (H_2SO_4) to each well. The plate was read at an OD of 492 nm using the Biotek Epoch Microplate Spectrophotometer.

Statistical analysis

GraphPad Prism 9 was used to conduct statistical analysis. Statistical significance was calculated using Mann-Whitney tests, Student *t* tests or 1-way analysis of variance (ANOVA) with Kruskal-Wallis tests when appropriate.

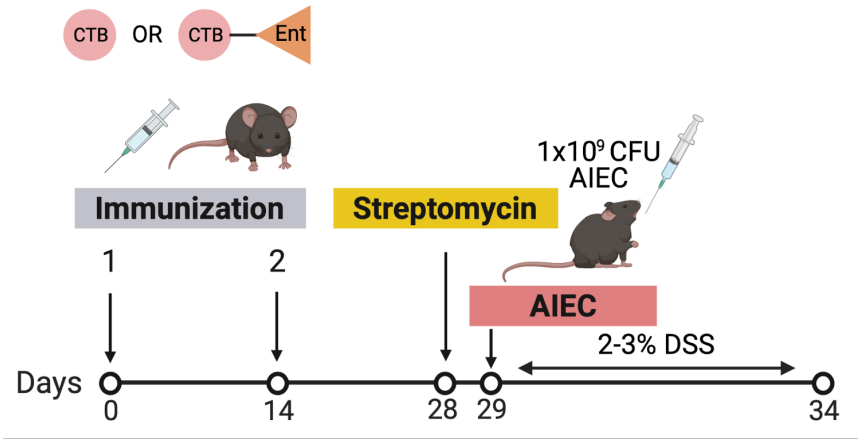


Figure 3. Schematic representation of experimental design for *Lcn2*$^{-/-}$ and WT mice. Mice were immunized with either CTB or CTB-Ent for the first 4 weeks. After complete immunization, the mice were orally gavaged with 20mg of streptomycin, followed by infection with 10^9 AIEC CFU 24h thereafter. Mice were provided with 3% DSS in the drinking water for the remainder of the experiment. Created with BioRender.com.

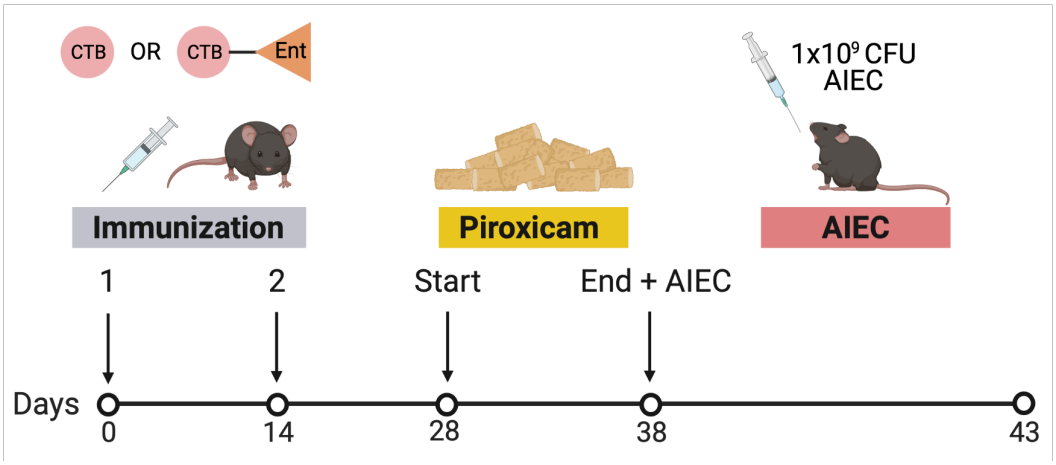
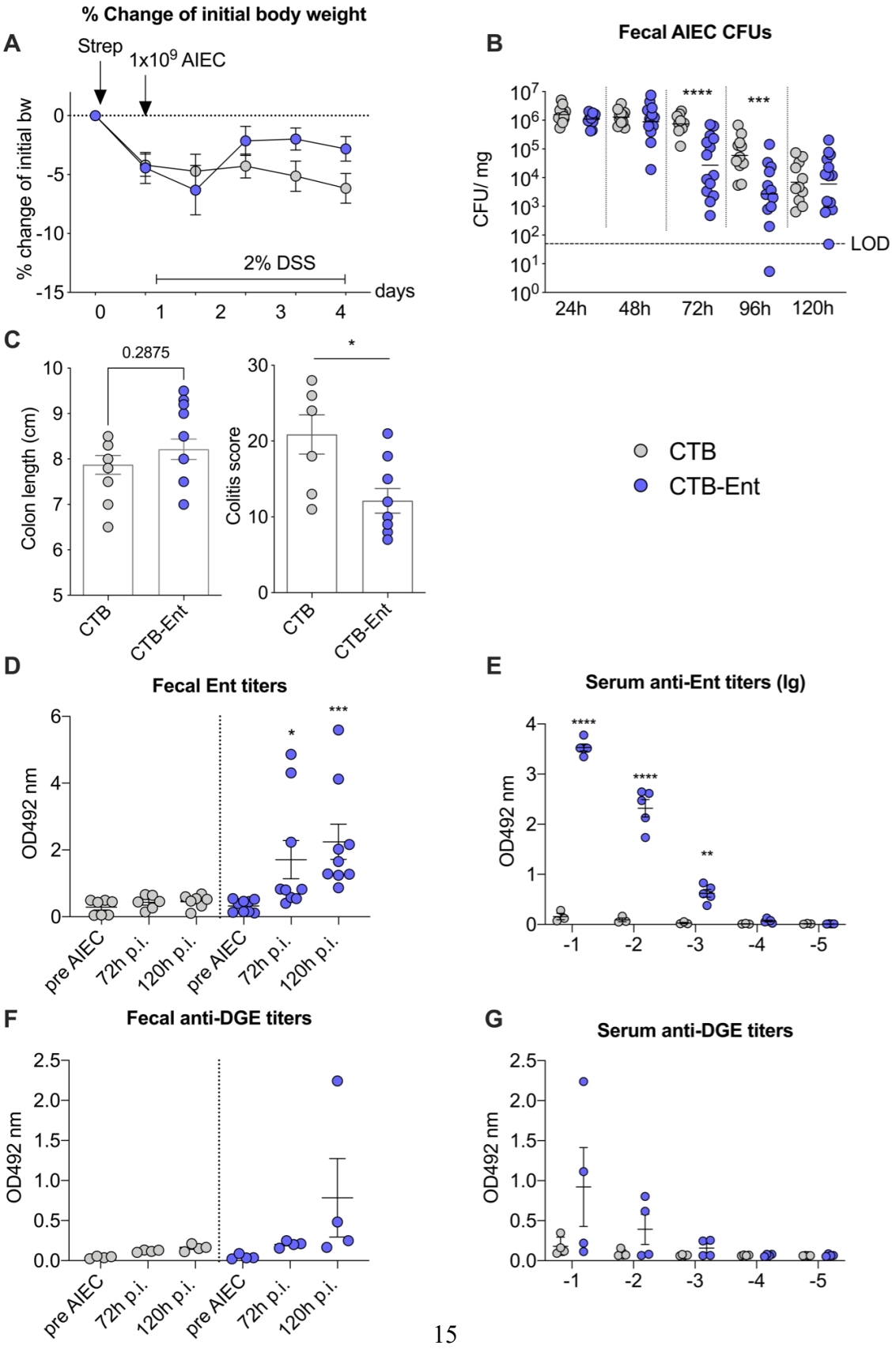


Figure 4. Schematic representation of experimental design for *Il10*$^{-/-}$ mice. Mice were immunized with either CTB or CTB-Ent for the first 4 weeks. After complete immunization, the mice were placed on a piroxicam-supplemented diet for 10 days. The mice were orally infected with AIEC and were placed on regular chow for the remainder of the experiment. Created with BioRender.com.

RESULTS

Figure 5. CTB-Ent immunization reduces AIEC colonization and ameliorates colitis in *Lcn2*$^{-/-}$ mice. *Lcn2*$^{-/-}$ mice were intranasally immunized with CTB (control group) or CTB-Ent (experimental group). After complete immunization, the mice were given streptomycin via oral gavage and were orally infected with 10^9 AIEC CFU per mouse the following day. Mice were provided with 2% DSS in the drinking water from the day of infection until the end of experiments. **(A)** Mean changes in body weight over time. **(B)** Geometric mean of AIEC CFUs per mg of feces over a period of 120 hours post-infection are shown in respective groups. **(C)** Mean colon lengths and histological colitis scores of respective groups 120h post-infection are shown (Longer colon length indicates less inflammation. Higher histological score indicates more severe colitis). **(D)** Fecal Ent titers pre-AIEC infection, 72 hours post-infection, and 120 hours post-infection are shown in the respective groups. **(E)** Mean serum anti-Ent titers (Ig) for samples 120h post-infection are shown in the respective groups. **(F)** Fecal anti-DGE titers pre-AIEC infection, 72 hours post-infection, and 120 hours post-infection are shown in the respective groups. **(G)** Serum anti-DGE titers of respective groups are shown 120h post-infection. Data represent mean ± SEM (A, C-G), or geometric mean (B). n= 4/group; ns, not significant; LOD, limit of detection; p.i., post-infection; * ($P \leq 0.05$); ** ($P \leq 0.01$); *** ($P \leq 0.001$); **** ($P \leq 0.0001$); unpaired student's t-test (A, C, D, E) or Mann-Whitney U test (B) was performed.

CTB-Ent immunization reduces AIEC colonization and ameliorates colitis in *Lcn2*$^{-/-}$ mice

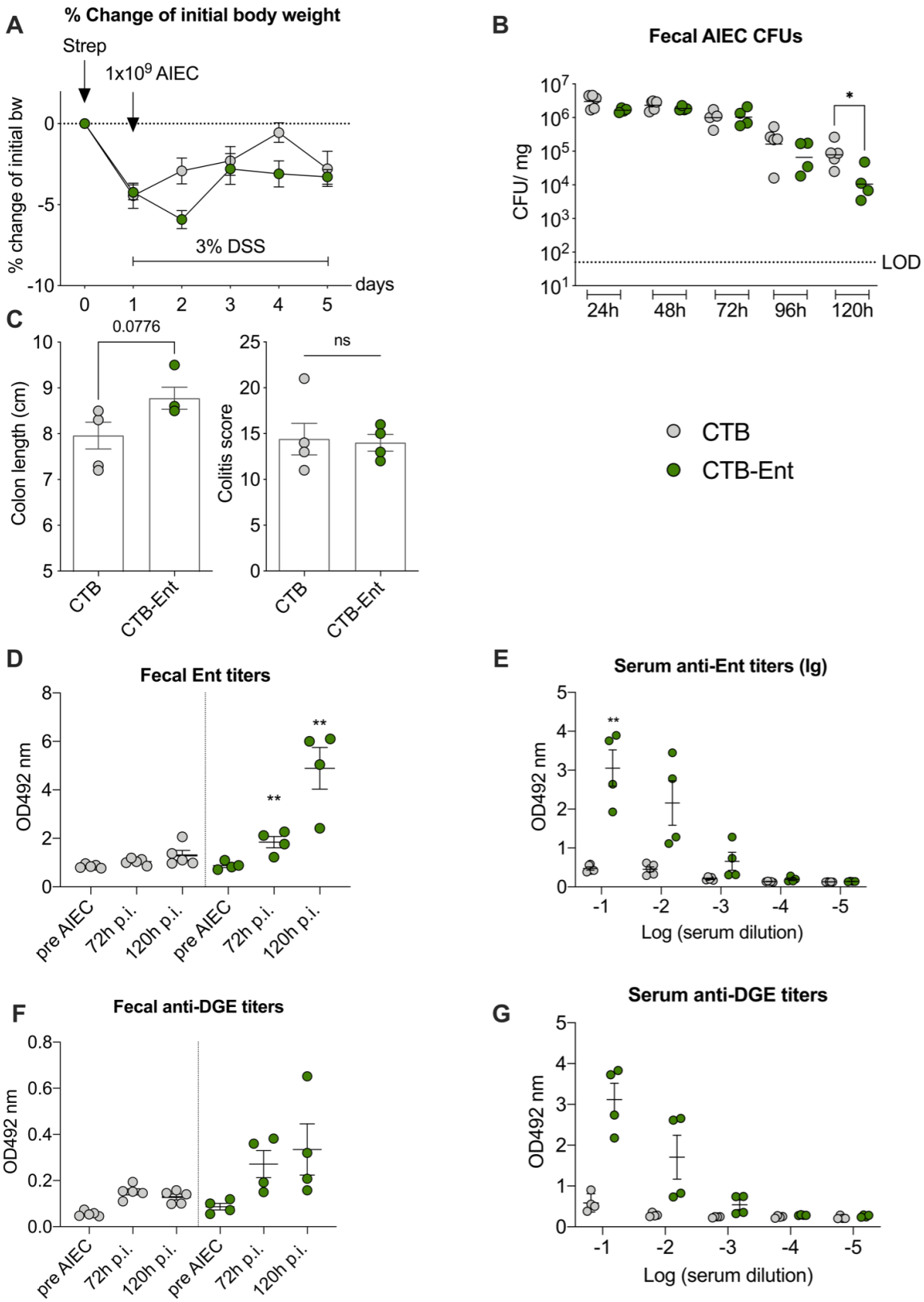


In order to assess the effectiveness of targeting siderophores in providing protection against AIEC colonization in *Lcn2*$^{-/-}$ mice, we used the CTB-Ent vaccination strategy developed by the Raffatellu and the Nolan lab. The CTB-Ent vaccine evokes a humoral immune response against the siderophore Ent and DGE. We first immunized *Lcn2*$^{-/-}$ mice with either the mock vaccine (unmodified CTB) or experimental vaccine (CTB-Ent) during the first 4 weeks. One day prior to infection, the animals received the antibiotic streptomycin, followed by oral infection with 10^9 AIEC CFU per mouse. Because AIEC is a human pathogen and does not colonize mice well in the absence of gut inflammation, and to mimic colitis observed in IBD patients, we provided the mice with 2-3% DSS to induce colitis and iron restriction, which would also trigger siderophore secretion by AIEC. Following infection, both groups of mice lost weight over the course of the AIEC infection (**Fig 5A).** CTB-Ent immunized animals appeared to make a recovery during the later days of infection, but this difference was not statistically significant **(Fig 5A)**. To assess the immunization efficacy in reducing bacterial colonization, we enumerated fecal AIEC shedding of both groups over time. At 24- and 48-hours post-infection (p.i.), both groups were similarly colonized. However, at 72h and 96h p.i., the CTB-Ent-immunized mice showed significantly fewer AIEC CFU as compared to the CTB control mice (72h p.i. $P \leq 0.0001$; 96 h p.i. $P \leq 0.001$), indicating that the immunization provided enhanced protection once the infection was established **(Fig 5B)**. Moreover, CTB-Ent-immunized mice had longer (less inflamed) colons in comparison to the CTB-immunized mice, although this difference was not statistically significant **(Fig 5C)**. Histologically, CTB-Ent mice had significantly lower colitis scores in comparison to CTB mice ($P \leq 0.05$) **(Fig 5C)**. After quantifying the presence of anti-Ent titers present in fecal supernatants, we saw a significant increase in titers in the CTB-Ent immunized group at 72h and 96h p.i. (72h p.i. $P \leq 0.05$; 96h p.i. $P \leq 0.001$) (**Fig 5D**). There was also a statistically significant increase in

total Ig anti-Ent titers in the serum of CTB-Ent immunized mice ($P \leq 0.0001$) **(Fig 5E)**. We also wanted to quantify the presence of anti-DGE titers in both the fecal samples and serum. Although there was a trend towards increased titers in the CTB-Ent mice, these differences were not significant **(Fig 5F, G)**. Collectively, these findings suggest that the CTB-Ent immunization provides protection against AIEC colonization by sequestering Ent, and thereby phenocopies Lcn2's role to neutralize Ent.

Figure 6. CTB-Ent immunization reduces AIEC colonization in WT mice. WT mice were intranasally immunized with CTB or CTB-Ent. After 4 weeks, mice were orally gavaged with streptomycin, followed by oral infection with 10^9 CFU/mouse of AIEC 24h later. Mice were provided with 3% DSS in the drinking water from the day of infection until the end of experiments. **(A)** Mean change in body weight over time. **(B)** Geometric mean of AIEC CFU per mg of feces over a period of 120 hours post-infection are shown in respective groups. **(C)** Mean colon lengths and histological colitis scores of respective groups 120h post-infection are shown. (Longer colon length indicates less inflammation. Higher histological score indicates more severe colitis). **(D)** Fecal Ent titers pre-AIEC infection, 72 hours post-infection, and 120 hours post-infection are shown in the respective groups. **(E)** Mean serum anti-Ent titers (Ig) for samples 120h post-infection are shown in the respective groups. **(F)** Fecal anti-DGE titers pre-AIEC infection, 72 hours post-infection, and 120 hours post-infection are shown in the respective groups. **(G)** Serum anti-DGE titers of respective groups are shown 120h post-infection. Data represent mean ± SEM (A, C-G), or geometric mean (B). n= 4/group; ns, not significant; LOD, limit of detection; p.i., post-infection; * ($P \leq 0.05$); ** ($P \leq 0.01$); unpaired student's t-test (A, C, D, E) or Mann-Whitney U test (B) was performed.

CTB-Ent immunization reduces AIEC colonization in wild type mice



To assess the efficacy of CTB-Ent immunization during AIEC infection in conditions that would recapitulate human patients (LCN2-proficient), we performed similar experiments with immunized WT mice. Following complete immunization, mice were administered streptomycin to disrupt the commensal microbiota, followed by oral infection with 10^9 AIEC CFU the following day. Mice received 3% DSS in the drinking water and were evaluated daily for weight loss and fecal shedding over the course of 120 hours after infection.

Similar to *Lcn2*$^{-/-}$ mice, both groups of mice began to lose weight after receiving streptomycin and continued to lose weight during the course of AIEC infection (**Fig 6A).** However, any differences between the two groups were not significant **(Fig 6A)**. Both groups were similarly colonized throughout the first days after infection, however, 120h p.i CTB-Ent-immunized mice had significantly fewer fecal AIEC CFUs as compared to CTB control mice ($P \leq 0.05$) **(Fig 6B)**. Furthermore, mice immunized with CTB-Ent showed a trend towards reduced colonic shortening (less inflamed) in comparison to the CTB mice, although this difference was not statistically significant **(Fig 6C)**. There was no significant difference in colitis scores between the two groups. We used an ELISA to quantify the fecal anti-Ent titers present in the samples from pre-AIEC infection, 72h and 120h post-infection. Immunization evoked a significant increase in Ent titers from the CTB-Ent mice during 72h and 96h p.i. in comparison to CTB mice (**Fig 6D)**. We also quantified the presence of anti-Ent titers (Ig) present in serum and found that there was a significant increase in total Ig titers in the CTB-Ent immunized mice (**Fig 6E)**. We also wanted to quantify the presence of anti-DGE titers in both the fecal samples and serum. Although there was a trend towards increased titers in the CTB-Ent mice, these differences were not significant **(Fig 6F, G)**. Overall, these results suggest that CTB-Ent immunization provides further protection even in the presence of Lcn2.

CTB-Ent immunization ameliorates colitis in *Il10*$^{-/-}$ mice

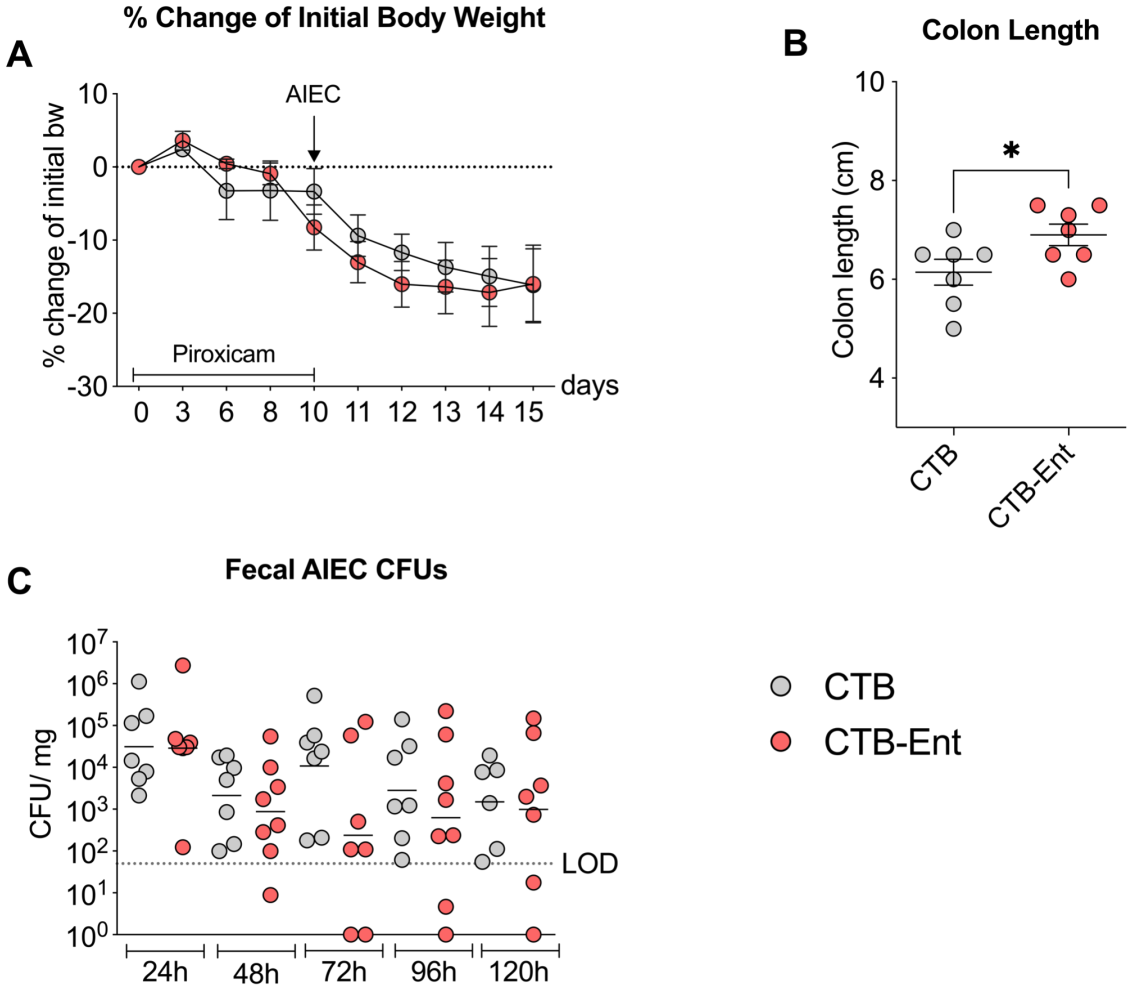


Figure 7. CTB-Ent immunization ameliorates colitis in *Il10*$^{-/-}$ mice. *Il10*$^{-/-}$ mice were intranasally immunized with CTB (control vaccine) or CTB-Ent (experimental vaccine). After complete immunization, the mice were placed on a piroxicam-supplemented diet for 10 days. On the last day of piroxicam, the mice were orally infected with 10^9 AIEC CFU per mouse and were placed on regular chow for the remainder of the experiment. **(A)** Mean change in body weight and **(B)** Mean colon lengths 120 hours post-infection are shown in the respective groups. (Longer colon length indicates less inflammation). **(C)** Geometric mean of AIEC CFU per mg of feces over a period of 120 hours post-infection are shown in the respective groups. **(D)** Mean anti-Ent IgA titers for fecal samples 24 hours and 96 hours post-infection are shown in the respective groups. Data represent mean ± SEM (A-B, D), or geometric mean ± SEM (C). n= 7-8/group; LOD, limit of detection; p.i., post-infection; * ($P \leq 0.05$); unpaired T test performed

In order to mimic AIEC infection in a genetic model for IBD, we immunized *Il10*$^{-/-}$ mice with either CTB or CTB-Ent during the first 4 weeks, and provided the mice with a piroxicam-supplemented diet for 10 days to accelerate colitis. Mice were orally infected with 10^9 CFU of AIEC, and the groups were evaluated for weight loss and fecal AIEC shedding over the course of infection (120h). Both groups started to lose weight during the piroxicam administration, which was further aggravated upon infection with AIEC. We did not observe differences in the weight course between groups of mice **(Fig 7A)**. To assess the immunization efficacy in reducing bacterial colonization, we enumerated fecal AIEC CFU during the course of infection. Although some CTB-Ent mice completely lost colonization between 72 hours to 120 hours post-infection, overall, differences between the two groups were not statistically significant (**Fig 7C)**. In order to assess potential differences in colon inflammation, we measured the colon lengths at the time of necropsy. CTB-Ent mice had significantly longer (less inflamed) colons in comparison to the CTB mice **(Fig 7B)**. The ELISA to quantify the anti-Ent IgA antibodies is currently in progress.

DISCUSSION

We established siderophore-based immunization as a promising strategy to induce anti-Ent/DGE antibodies, to confer protection against AIEC colonization, and to limit colonic inflammation in mice. We aimed to assess the efficacy of CTB-Ent immunization in **(1)** blocking the bloom of *Proteobacteria* and **(2)** providing protection against AIEC colonization. Fecal samples collected during various time points including pre-immunization, post-immunization, pre-AIEC infection, and post-AIEC infection are currently processed for 16S rRNA sequencing. This analysis will allow us to identify the impact of CTB-Ent immunization onto different species of bacteria present in the gut microbiota and compare the relative abundance of bacterial species between the two groups (Rapin et al., 2017). A previous study has even shown that CTB-Ent-immunized mice revealed expansion of beneficial microbes such as *Lactobacillus* spp. (Sassone-Corsi et al., 2016). In our first aim, we planned to analyze the sequencing data to test whether our immunization strategy would alter the bloom of *Proteobacteria,* which frequently utilize siderophores such as Ent. Although we do not have these data ready yet due to time limitations, this analysis will be included in our manuscript which is currently in preparation.

We used three different mouse models to assess the effects of CTB-Ent immunization in providing protection against AIEC colonization, our second aim. We started our studies with *Lcn2*$^{-/-}$ mice, because these mice are deficient in lipocalin-2, the evolutionary conserved antimicrobial peptide that targets Ent and thereby prevents bacterial iron uptake. Thus, *Lcn2*$^{-/-}$ mice are more susceptible to bacterial infection (Flo et al., 2004). We found that in the absence of this crucial antimicrobial protein, CTB-Ent immunization provided substantial protection against AIEC colonization, as demonstrated by the significantly reduced AIEC CFUs in comparison to CTB control mice **(Fig 5B)**. Even though differences in colon lengths were not significant, the

CTB-Ent immunized mice displayed less severe colitis as determined by histological scoring **(Fig 5C).** These results suggest that the CTB-Ent immunization provides a similar protection that lipocalin-2 would naturally, validating our goal of creating a vaccine that would mimic the role of lipocalin-2 and potentially neutralizing abilities towards DGE, although this needs to be followed up on.

Human immune cells transcribe, translate, and secrete lipocalin-2 during inflammation and upon encountering bacterial siderophores. It could be argued that immunization against bacterial Ent would be redundant to the host's LCN2. To test this, we assessed the effects of CTB-Ent immunization in Lcn2-proficient WT mice. We found that the immunized mice still displayed a significant decrease in AIEC CFUs at the end of experiments and had a trend of reduced colonic inflammation (**Fig 6B, C**). Moreover, the CTB-Ent mice had significantly higher anti-Ent titers in both fecal samples and serum in comparison to the CTB mice, suggesting that our CTB-Ent immunization provides further protection even in the presence of Lcn2. Our hypothesis is that this is due to antibodies that cross-react to DGE.

Finally, we immunized *Il10*$^{-/-}$ mice as a genetic mouse model for IBD. The CTB-Ent immunized mice had significantly decreased colonic shortening, suggesting that the immunization may have ameliorated colitis. The analysis of additional parameters such as histological colitis scores and Ent/DGE titers are currently in progress. It is important to note that intestinal inflammation is a prerequisite in order to maintain sufficient AIEC colonization. The piroxicam-accelerated model of colitis induces severe intestinal inflammation. Thereby, we could mimic similar conditions as those seen in IBD patients, who suffer from chronic intestinal inflammation. Our established model of colitis allows for the expansion of AIEC, which uniquely thrives in an inflamed gut and might thereby provoke even more inflammation. (Zeng et al., 2017). We

hypothesized that the CTB-Ent immunized *Il10*$^{-/-}$ mice would have a significant decrease in AIEC colonization. Although we observed a strong decrease in AIEC shedding in some of the CTB-Ent mice (**Fig 7C)**, there was also some variation between the mice as expected. This is most likely due to differences in colonic inflammation following piroxicam administration. Interestingly, 5 out of 8 CTB-Ent immunized mice were housed together in the same cage, and these same mice were all collectively losing more weight already during piroxicam and had higher AIEC colonization. One possibility is that there could have been a super-shedder among these mice that impacted the rest of the group. For future studies, we will house fewer mice in cages to hopefully obtain more consistent results.

In contrast, the streptomycin/DSS model utilized for WT and *Lcn2*$^{-/-}$ mice shows less variation in terms of AIEC colonization because streptomycin disturbs the commensal microbiota and creates a niche for AIEC to colonize. DSS induces colonic inflammation that triggers AIEC to secrete siderophores to counteract the host-mediated iron restriction. However, this model is not ideal for analysis of the microbiota because the employed antibiotic as well as DSS would mask the true effect of siderophore immunization onto the native microbial composition. This is why we still think that the piroxicam-accelerated colitis model is superior to the latter, but we will have to increase the number of mice per group to make a conclusion. For the future, our short-term goals include performing histological analysis of H&E-stained colon tissues in order to compare intestinal inflammation between the CTB immunized and CTB-Ent immunized mice. We have already preserved colon sections from all the mice to process for histology. We will also repeat the *Il10*$^{-/-}$ immunization study and use different housing conditions to see if we can achieve more consistent colonization results. Another goal is to perform bacterial fluorescence in situ hybridization (FISH), which is a technique used to detect the presence of specific groups of

microorganisms within the mucus-preserved natural colonic habitat. This will give us a better idea as to whether the presence or absence of siderophore antibodies results in differences of AIEC colonization and adherence pattern to the intestinal epithelium. Notably, commensal-specific IgA has been shown to promote the clearance of microbial antigens and pathogenic microorganisms from the gut by blocking their adhesion to epithelial receptors.

As mentioned earlier, we have also preserved fresh fecal samples from different time points for 16S rRNA sequencing that will allow us to compare potential changes in microbiota composition. For our long-term goals, we aim to isolate Ent-specific B cells from mice to generate monoclonal anti-Ent/DGE antibodies. These will be cloned into immunoglobulin (antibody) vectors for the mass production of humanized anti-Ent/DGE antibodies. Monoclonal antibody therapy is a known, yet underdeveloped strategy for microbial diseases (Saylor et al., 2009). Successful generation of humanized Ent-antibodies will provide a novel and narrow-spectrum antimicrobial approach to target siderophore-dependent pathogens in human diseases including IBD.

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