Investigating Immune Markers of Cannibinoidal Virulence in Enteric Pathogens

(Student name) - Mellies Lab

Summer 2021

Abstract

Escherichia coli are a Gram-negative bacterium that normally live in the intestines of healthy people. Most strains of *E. coli* are harmless or cause brief and mild cases of diarrhea. But there are a few types of *E. coli*, such as enterohemorrhagic *E. coli* (EHEC) O157:H7, which causes symptoms such as more serious stomach cramps, bloody diarrhea, and vomiting. EHEC is found in the intestines of cattle and transmitted to humans through consumption of food or water that had been contaminated. EHEC can also cause hemolytic uremic syndrome, which lead to kidney failure and death.

EHEC cannot be treated by traditional antibacterial methods as it contains a toxin that is very similar to a well-known Shiga toxin produced by *Shigella dysenteriae* type 1. Shiga toxin is one of the most potent biological poisons known. Traditional antibiotic methods work by breaking the bacteria open and stopping the bacteria from further colonization by degradation. But in bacteria that contain Shiga toxin or Shiga-like toxin (like EHEC), when the bacteria are lysed, the toxin is released into the bloodstream of the host and causes acute symptoms that are often worse than the symptoms caused by the bacteria itself. Due to this, EHEC cannot be treated with antibiotics. Currently there are no treatments for EHEC besides support and management of symptomes.

Current research into new ways to treat EHEC has found something called 2-arachidonoyl glycerol (2-AG), an endocannabinoid that is produced in the human intestinal tract that has been shown to reduce the virulence of EHEC. 2-AG is very similar to Cannabinoidal (CBD) which lead the Mellies Lab to begin to look into the effect of CBD on EHEC. It has been seen that in vitro CBD decreases the secretion of a protein called espA, which is needed to move EHEC around the intestine. A mouse model using *Citrobacter rodenium* showed that there is a reduction in colonization when the mice were treated with CBD as well. To further understand this reduction in colonization by CBD, we wish to use different immune markers that tell us more about what genes are being affected by the CBD by using a qPCR (quantitative polymerase chain reaction). This will allow us to measure the amounts of different genes in the tissue samples and paint a fuller picture of the true effect CBD is having on the immune system in the mice, which can then be applied to EHEC. Understanding this picture gets us closer to being able to use CBD in clinical settings as a treatment for EHEC and other gram-negative bacteria.

Background and Rationale

Escherichia coli are Gram-negative bacteria with many strains and variants (1). We are interested in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a strain of E coli that resides primarily in the recto anal junction of cattle and is transmitted to humans through consumption of food or water that was contaminated from cattle manure (2). EHEC then colonizes the epithelial tissue of the intestines through A/E lesion formation, causing gastroenteritis. A/E lesions are characterized by the bacteria tightly attaching to the enterocyte surface, and both localized destruction of microvilli and huge ultrastructural changes underneath the bacteria from

the accumulation of filamentous actin (3). EHEC is known to stimulate an inflammatory response in the intestine through host recognition of flagellin and lipopolysaccharide (LPS) (7). Flagellin and LPS are a part of the innate immune system which is the first line of host defense against pathogens. EHEC's primary site of infection with the attaching and effacing (A/E) pathogens is the epithelium lining of the mucosal surface of the gastrointestinal tract. These epithelial cells play many roles including ion transport, fluid uptake, and secretion that are critical to maintaining homeostasis in the digestive system. Additionally, the epithelial cells coordinate the expression and upregulation of specific antimicrobial products in response to infection. When the epithelial cells become infected, these pathways become disrupted (7). An example of this is that EHEC is capable of inhibiting the gamma interferon (IFN-γ) proinflammatory pathway through inhibiting Stat-1 phosphorylation. The IFN-γ pathway is important for host defense and as this inhibition allows EHEC to spread further in the host (8).

Symptoms of EHEC infection include abdominal cramps, severe bloody inflammatory diarrhea, fatigue, nausea, Hemolytic uremic syndrome (HUS), and even death. HUS is a condition that occurs when the small blood vessels in kidneys become damaged and inflamed and causes clots to form in the vessels and can lead to kidney failure and death (4, 5). EHEC is cannot be treated by traditional antibiotics due to a toxin called Shiga like toxin, Stx1, which is released into the bloodstream when EHEC bacterial cells are lysed by traditional antibiotics, and causes acute symptoms like HUS (6) that are worse than the symptoms from the EHEC itself.

While there are no current treatments for EHEC, recent research (9) has shown that 2-arachidonoylglycerol (2-AG), an endocannabinoid produced in the human intestinal track, reduces the virulence of EHEC. 2-AG is a host lipid hormone that can modulate gut biology. They have specifically found that mice with increased 2-AG are protected from enteric bacterial infection, that 2-AG inhibits virulence in EHEC and *C. rodentium*, and that 2-AG antagonizes the bacterial pro-virulence receptor QseC to decrease virulence. 2-AG is very similar to Cannabinoidal (CBD), a non-psychotic phytocannabinoid. In vitro testing has revealed that CBD causes a concentration dependent decrease on the secretion of espA, a thread protein that is integral for the translocation and delivery of EHEC virulence factors (10). In-vivo testing of CBD in a mouse model using *C. rodenium* additionally showed that there is a reduction in colonization of the gut corresponding to CBD administration in the mouse model. This is also seen when using the FDA-certified cannabinoidal pharmaceutical Epidiolex (11, 12). The molecular mechanism of this reduction in EHEC virulence by CBD was investigated using RNA-seq and while the results were unclear, it seems like the stress response genes had altered expressions (13) in the tissue treated with CBD and this will be investigated further in the future.

The effect of CBD on intestinal immunity is actively being studied as for CBD to be used clinically, the effect of the medication itself needs to be understood. It has been found that CBD is immune suppressive and anti-inflammatory. The mechanism by which CBD acts is through direct suppression of activation of immune cells and promotion of regulatory cells, which then controls other immune cell targets. Critical targets of suppression include cytokines, including IL-1β, which we wish to investigate in this project (14).

If CBD is capable of reducing pathogenicity of gram-negative bacteria such as EHEC and *Citrobacter rodenium* we could begin to clinically treat EHEC. It also has the potential to treat *Salmonella* infections, where there are also no current effective treatments. In addition, there is an antibiotic crisis in which 2.8 million people in the United States develop antibiotic resistance infections every year and scientists are running to find new ways to treat them (15). If CBD can

reduce pathogenicity in a wide range of gram-negative bacteria, we can have a wider variety of tools to treat these bacteria that have gained antibiotic resistance.

We want to further investigate the immune markers of gut tissue harvested from the mice that were treated with both Epidiolex and CBD using qPCR. This will allow us to further understand the large immune impact that the Epidiolex and CBD are having on *C. rodenium* in the mouse model. The qPCR results will also allow us to begin to unravel the roles of the immune response, metabolism, translation, and stress pathways in moderating the EHEC's virulence in response to CBD. The specific immune markers we have selected are: IL-1β, a signal used for pro-inflammatory activation of M1 macrophages activated by LPS that is also responsible for clearance of *C. rodenium* from healthy hosts (16), INF-γ, another pro-inflammatory signal that is linked to weight loss in mice exposed to *C. rodenium* (17), IL-10, an anti-inflammatory that has when not present in mice, they exhibit weight loss and IBS symptoms due to bacterial infiltration (3), TLR4, a Toll-like receptor for Gram-negative bacteria that induce NF-kβ, a transcription factor that involves many inflammation and epithelial proliferation genes, and NOD-1, a receptor that recognizes gram-negative bacteria and signals IL-1β and NF-kβ (18).

Specific aim and hypothesis

Our specific aim is to identify the affect that CBD is having in the *C. rodenium* through the genetic regulation of the immune markers we have selected. When we have a better picture of the immune response, then we can work to understand the mechanism of CBD infiltration into the bacteria and the virulence reduction mechanism.

I hypothesize that IL-1β, INF-γ, and TLR4 will be upregulated in the treated tissue compared to the untreated tissue, marking the improved immune response through mediation of inflammation and clearance of *C. rodenium*.

Design and Procedure

We will use qPCR to look at a variety of immune markers in gut tissue harvested from mice treated with both Epidiolex and CBD. The infection has already been conducted (Maddox Zhang, thesis '20 in collaboration with Tim Nice at OHSU). The tissue has been harvested, RNA isolated and cDNA synthesized and is currently stable in -80 freezer. qPCR allows for determination of the initial number of copies of template DNA with accuracy and analyze both qualitatively and quantitatively. We will use the qPCR data to allow us to compare the differential expression of the immune markers in the cecal tissue samples. This will allow us to see a larger picture of the differential gene expression in different stages of treatment.

Predicted and alternative outcomes

My predicted outcome is that IL-1β, NOD1, and TLR4 will be upregulated in the intestinal tissue treated with CBD or Epidiolex compared to the untreated tissue. This would mark an improved immune response through the mediation of inflammation and clearance of the bacteria.

It is completely possible that the other markers we are looking at, IL-10 or NF-kβ, are what are upregulated instead. It would be surprising if NF-kβ was upregulated but TLR-4 was not, as TLR-4 induceds NF-kB, but so little is currently known about the effect of CBD on the regulation of the *C. rodenium*. My hypothesis is based on the fact that it has been seen that TLR-4 mediates inflammation and clears bacteria load, IL-1β regulates the cytokine balance which

certain cytokines are involved in inflammatory responses as well, and INF-γ is also an inflammatory response and should be upregulated like the others. Some of these markers could also be downregulated, not upregulated, and beginning to see which ones are doing what will allow us to see more the whole picture of what is going on. Any information about the differential expression will allow us to make more informed hypothesizes about the system.

There is also a non-zero chance that these are not the pathways that are affected by the CBD, in which case we would return to the literature to find new immune markers to look at.

Role of Student
As the student I will be conducting the research in the lab, working with Jay to create protocols and analyze not only the data we collect, but additionally the data for that has been previously collected. I will also be working to write up all we have learned about this system in an effort to publish as a co-author on the manuscript. We will also be writing a NIH R21 grant submission together.

Role of Faculty
Jay Mellies will supervise the conduction of research, including the writing of procedures, the research done before lab work is done, and the day-to-day progression. He will also be a co-author on the manuscript and will be helping write the NIH R21 grant submission.

Benefit to student (written by Jay Mellies)
Funding this project will benefit the student in multiple ways. First, it is a logical extension of interests developed in the Microbiology course, spring 2020. Unfortunately, because of the pandemic the student was not able to work on the project with me this past summer. However, for summer 2021, she will conduct hypothesis-driven inquiry on the topic, solidifying her understanding of the scientific method, also contributing to the writing of a manuscript, as well as an NIH grant submission. Through the project, the student will also gain a fundamental understanding of mucosal immunity. In sum, these opportunities fit well with the student's career aspirations of acquiring graduate training in molecular pathogenesis, and the intersection of basic and applied research efforts that ultimately lead to improving human health.

Citations:

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Research Supply Budget
RCSRF Application
(Student name)
Mellies Lab

Materials needed:
Primers → $129.09 from ThermoFisher

- 8 pairs → 16 total

SYBR Green Mix → $417 from ThermoFisher

- Found here https://www.thermofisher.com/order/catalog/product/A25780?ICID=search-product?ICID=cvc-sybr-realtime-c1b1#/A25780?ICID=search-product%253FICID
- Contains SYBR Green dye, Dual-Lock Taq DNA Polymerase, dNTPs with dUTP/dTTP blend, heat-labile UDG, ROX passive reference dye, and optimized buffer components.
- Contains 5 X 1 mL tube, sufficient for 500 20-µL reactions
- As we have 8 genes to amplify including the 2 reference genes, 500 total reactions will be sufficient

24-well qPCR plates → $128 for 50 plates

- 50 plates will allow for 1200 reactions to be run, which is sufficient

SuperScript III First-Strand Synthesis Invitrogen ImmunoMix → $588 for 500 reactions

- Found here https://www.thomassci.com/Chemicals/PCR-Reagents/_/ImmoMix

Invitrogen UltraPure Agarose → $198

- Found here https://www.thermofisher.com/order/catalog/product/16500100#/16500100

Total request $1460