Kitts, Lauren

Research Plan
The Effect of Diet on Immune Cells in Humanized Gnotobiotic Mice Colonized with IBD Microbiomes
Biomedical and Health Sciences

Rationale

Inflammatory Bowel Disease (IBD) is an autoimmune disorder that affects the digestive tract. It is an increasingly prevalent chronic disease and affects over 1 million people in the United States alone and millions more globally. Cases of Inflammatory Bowel Disease are increasing rapidly, and since there is no cure, once the disease begins, patients are riddled with it and its problems for life. This costs patients with IBD approximately 3 times the amount that healthy patients spend on healthcare a year, creating an enormous financial burden (Rapaport, 2019). Under the umbrella term of IBD, there are two main sub-diseases, which are Crohn's Disease and Ulcerative Colitis. There are small differences between the progress of disease between Crohn's Disease and Ulcerative Colitis, but they are often studied together because of their striking similarity. Inflammatory Bowel Disease is not a curable disease at this point in time, and treatments currently available have a variety of negative side effects, which mean that they are not a long term solution to the increasing problem. Additionally, the inflammation caused by IBD can lead to other diseases later in life, such as colon cancer. This disease has also been linked with an increased risk of heart disease. The etiology of Inflammatory Bowel Disease has previously been linked to its interactions with the gut microbiome, a community of commensal bacteria which reside in the digestive tract. The microbiome's relationship with IBD is thought to be the primary cause of the disease. Previous research has shown that diet has an impact on the microbiome, and on IBD as a whole, meaning that it could possibly become a new treatment. A study done by Llewellyn et al found that psyllium, a type of fiber, had a positive

effect on immunity while casein had a negative effect on immunity. This means that the types of food eaten have specific effects on the microbiome, furthering the theory of a dietary cure for IBD. Additionally, a study by Britton et al demonstrated that in patients with IBD, Th17 and RORgt+ Treg cells were found in different proportions compared to non-IBD patients. Specifically, IBD patients had increased levels of Th17 cells and decreased levels of RORgt+ Treg cells. This study demonstrated that the immune system is different in IBD patients versus normal patients. However, the IBD community doesn't know whether the effects of the diets demonstrated in previous studies are affected by different specific microbiomes derived from patients with and without IBD. Therefore, more research is necessary to determine the reason for its effect before diets can be used to treat humans with IBD.

Research Question

- What is the impact of the specific microbiomes on the effects of a diet high in protein in mouse models?
- What is the impact of the specific microbiomes on the effects of a diet high in fiber in mouse models?

Hypothesis

- If mice colonized with IBD microbiomes are put on a diet high in casein, the diet will have a significant effect on the levels of inflammatory immune cells. This will show that the pro-inflammatory effect of a protein-based diet is unchanged by an inflammatory microbiome since previous research has shown that diets high in protein were successful in increasing inflammatory markers in mice with IBD (Llewellyn, 2018).

- If mice colonized with non-IBD microbiomes are put on a diet high in psyllium, the diet will not have a significant effect on the levels of inflammatory immune cells. This will show that the anti-inflammatory effect of a fiber-based diet is decreased by a non-inflammatory microbiome, since previous research has shown that non-IBD microbiomes do not increase immune cell levels, meaning the diet would not have a significant effect in decreasing the levels of immune cells. (Britton, 2019; Llewellyn, 2018).

Engineering Goal

- There are no engineering goals for this project.

Expected Outcomes

- It is expected that diets high in fiber in mice colonized with a non-IBD microbiome will show less of an effect than seen in previous research because the diet works to decrease the levels of immune cells, and the microbiome does not create additional immune cells. Additionally, it is expected that diets high in protein will show a significant effect on the levels of immune cells in mice colonized with IBD microbiomes. This is because the IBD microbiome will cause more immune cells to be in the mesenteric tissue, and the diet will work to increase the levels of these cells. The effects of diet are expected to be influenced by microbiome because there have been previous studies linking diet and the microbiome, and the microbiome and immune function (Llewellyn, 2018; Britton, 2019; Turnbaugh, 2009). If this is the case, then diets high in protein or fiber will be significantly impacted by microbiome type, which will be seen in the levels of Th17 and

RORgt+ Treg cells. However, if the hypothesis is refuted, there will be a negligible change in the levels of immune cells across the microbiome types.

Procedure

Role of Mentor

Mentor will provide mouse tissue samples and materials for the experiment. Additionally, the mentor/designated supervisor will provide guidance in the goals and direction of the project, and assist with the analysis of final results. The mentor/ designated supervisor will gavage and colonize the mice, switch the diets of the mice from regular mouse chow to the experimental diets, and sacrifice the mice after 14 days. The mentor/designated supervisor will provide excess colon, small intestine, and lymph node tissue for the student to analyze through flow cytometry after the mice have been sacrificed for the mentor's study. Tissue samples will be extracted from the mouse by the mentor/designated supervisor and subsequently given to the student to prepare and stain. Additionally, the mentor/designated supervisor will assist in the set up of the flow cytometry device and provide software with which to analyze the resulting data.

Role of Student

Tissue preparation

- Colon tissue will be wetted in phosphate-buffered saline solution
- One pellet of fecal matter will be collected from the colon using forceps, the rest will be discarded
- Colon will be cut open vertically and the epithelial layer will be scraped
- Colon tissue will be placed in a tube containing a phosphate-buffered saline solution and brought to the lab
- Small intestine tissue will be wetted in phosphate-buffered saline solution
- Peyer's patches will be removed to prevent false positives

- The small intestine will be cut open and epithelial layer will be scraped
- Small intestine tissue will be placed in a tube containing a phosphate-buffered saline solution and brought to the lab
- Lymph nodes will be placed in phosphate-buffered saline solution

Tissue digestion

- Each tissue will be transferred to a 'Tube 2' containing 10 ml of HBSS
- Vortex tissue briefly
- Place each tissue into a 'Tube 3' containing 10 ml of dissociation buffer
- Shake in the incubator for 30 minutes at 37°C at 110 RPM on a shaker.
- Vortex after 15 minutes in the incubator
- Remove tissue samples from incubator and vortex
- Transfer tissue samples to a 'Tube 4' containing 20 ml cold HBSS and vortex
- Cut each tissue sample into pieces with scissors and transfer to a 'Tube 5' along with 10 ml prewarmed Digestion Buffer
- Shake at 110 rpm at 37°C for 30 minutes, and shake the tubes by hand halfway through
- Pipette samples from 'Tube 5' set into a filtered 'Tube 6' which will contain 10 ml cold RPMI and wash strainer with 10 ml cold RPMI
- Centrifuge cells from 'Tube 6' at 1500 rpm, 4°C for 5 minutes
- Vacuum supernatant and wash once in 10 ml RPMI

Staining and Flow Cytometry

- Lymph nodes will be placed on filters and manually forced through small filters to prevent fat or large particles from entering the sample and then stained with antibodies
- Divide all samples into two 96 well plates
- On plate 1, pipette antibodies to show myeloid cells
- On plate 2, pipette antibodies to show T cells
- Pipette up and down in both plates to mix the antibodies and the samples
- Pipette each sample into the flow cytometry machine, making sure to rinse and set each parameter to get accurate data

- Collect data and analyze

Risk and Safety

- Chemicals used during this study are not harmful and any spillage or ingestion will be reported and dealt with appropriately. All chemicals will be handled with protective clothing and gloves.
- Pipettes will be handled with care to prevent scratches and gloves and proper clothing will be worn at all times. Additionally, used pipette tips will be placed in sharps containers.
- Tools used in the preparation of colon, small intestine, and lymph node tissue will be handled with care and protective equipment will be utilized to prevent injury.
- During the preparation of colon, small intestine, and lymph node tissue, face masks will be utilized to prevent inhalation of murine materials such as fur.
- Murine fecal matter will be handled with gloves and contained in biohazard cabinets to prevent inhalation of bacteria or contamination. It will be placed in airtight containers and stored in the fridge.
- Clidox used during the sanitation of cages and workspaces will be handed using proper equipment and clothing. Any exposure will be washed with water and ethanol.

Data Analysis

Data analysis during this study will consist of using flow cytometry data and comparing percentages of cells that have been stained to determine the overview of immune tone. This will be done using FloJo on a Cytek Aurora flow cytometry machine.

Bibliography (APA)

Britton, G. J., Contijoch, E. J., Mogno, I., Vennaro, O. H., Llewellyn, S. R., Ng, R., . . . Faith, J. J. (2019). Microbiotas from Humans with Inflammatory Bowel Disease Alter the Balance of Gut Th17 and RORγt Regulatory T Cells and Exacerbate Colitis in Mice. *Immunity,50*(1). doi:10.1016/j.immuni.2018.12.015

Chen, L., He, Z., Iuga, A. C., Filho, S. N., Faith, J. J., Clemente, J. C., . . . Lira, S. A. (2018). Diet Modifies Colonic Microbiota and CD4 T-Cell Repertoire to Induce Flares of Colitis in Mice With Myeloid-Cell Expression of Interleukin 23. *Gastroenterology,155*(4). doi:10.1053/j.gastro.2018.06.034

Llewellyn, S. R., Britton, G. J., Contijoch, E. J., Vennaro, O. H., Mortha, A., Colombel, J., . . . Faith, J. J. (2018). Interactions Between Diet and the Intestinal Microbiota Alter Intestinal Permeability and Colitis Severity in Mice. *Gastroenterology,154*(4). doi:10.1053/j.gastro.2017.11.030

Mouse care. (2014, August 20). Retrieved from https://www.animalhumanesociety.org/adoption/mouse-care

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., & Gordon, J. I. (2009). The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Science Translational Medicine,1*(6). doi:10.1126/scitranslmed.3000322

Vertebrate Animal Research

a. There are no alternatives to vertebrate animal use in this project because it requires diets to be fed and is based on the reaction of the microbiome and immune systems to said diets, which can not be seen without the immune system or microbiome. The immune system and microbiome only reside in vertebrate animals for the purposes necessary in this study. Cell cultures would not be a viable method of testing this since they do not contain microbiomes and complex immune systems. This means that it is necessary to use vertebrate animals in order to conduct this research.
b. This research will be very impactful to the Inflammatory Bowel Disease research community because the knowledge about the interactions between diet, the microbiome, and the immune system could lead to possible treatments and increase the community's understanding of Inflammatory Bowel Disease as a whole. Specifically, the knowledge of the cause behind the two diets' effects could lead to further tailoring of a treatment diet for IBD, since the majority of current dietary treatments are based on elimination diets and not on previous research.
c. Mice will be treated using specific procedures to minimize potential discomfort, distress, pain, and injury
    i. Mice will be scruffed to prevent injury or discomfort, gavaged and given 1-2 ml of a fecal cocktail by the mentor.
    ii. Mice will be humanely euthanized at the end of the study to examine their tissues. Euthanization will be done by the mentor using CO_2 gas.
d. There will be 24 male mice used in this study. The mice will be C57BL/6 mice, age 6 weeks at the beginning of the study, and will be from the Mount Sinai

Gnotobiotic Facility. 24 mice will be used to prevent anomalies from seeming significant. Mice will be used in accordance with IACUC protocol LA13-00058.

e. Mice will be housed in 3 mouse cages since mice are social animals. Mice will be separated by the experimental diet that will be used. Mice will be monitored daily to ensure consistent health.

f. At the termination of the study, the mentor/designated supervisor will humanely euthanize mice using CO2 gas and harvest tissue necessary for detailed analysis

2. Potentially Hazardous Biological Agents Research

a. Cultured microbes will be sourced from human fecal matter by the mentor. Additionally, mouse tissue will be collected from 24 mice by mentor and prepared for flow cytometry by the student. BSL level was determined to be BSL2 by Mount Sinai.

b. Murine fecal pellets will be handled with protective clothing and gloves and contained inside biohazard cabinets or airtight containers.

c. Safety precautions included protective clothing such as close-toed shoes, gloves, lab coats, caps, gowns, shoe covers. Additionally, potentially hazards will be handled in fume hoods or biohazard cabinets to prevent inhalation of fumes or bacteria. All sharp objects will be disposed of in designated sharps bins. Any hazardous material will be disposed of in the marked biohazard bins, which are disposed of by Mount Sinai.

3. Hazardous Chemicals, Activities, and Devices

a. The risk of the laboratory is minimal due to the small amount of hazardous chemicals present. Any chemicals that have the potential to be hazardous are stored in the fume hood and are only used with supervision at the fume hood. All containers meet fire and safety codes in the laboratory.
b. The student will be supervised by the mentor or the designated supervisor when the student is handling potentially hazardous chemicals.
c. Safety procedures will include proper clothing and using fume hoods to limit exposure during any processes that could cause potentially hazardous chemicals to be available.
d. Chemicals will be disposed of using the waste bins specifically labeled with the chemical. They will then be disposed of by Mount Sinai.