UC Riverside

UCR Honors Capstones 2020-2021

Title

Sex-Dependent Effect of Lps-Induced Inflammation on Claudin-3: Murine Jejunum

Permalink

https://escholarship.org/uc/item/8zt9906g

Author

Murad, Maryam

Publication Date

2021

Data Availability

The data associated with this publication are within the manuscript.

SEX-DEPENDENT EFFECT OF LPS-INDUCED INFLAMMATION ON CLAUDIN-3:
MURINE JEJUNUM

By

Maryam Murad

A capstone project submitted for Graduation with University Honors

March 11th, 2021

University Honors
University of California, Riverside

APPROVED

Dr. Monica J Carson
Division of Biomedical Sciences

Dr. Richard Cardullo, Howard H Hays Jr. Chair
University Honors

Abstract

The small intestine—consisting of the Duodenum, Jejunum, and Ileum—plays a vital function in breaking down food from the stomach and absorbing nutrients. The jejunum, specifically, functions in absorbing fatty acids, sugars, and amino acids. The jejunum is investigated using NanoString-based analysis of gene expression to determine the response of lipopolysaccharide (LPS) induced inflammation in male and female murine. In addition, the preliminary data demonstrated a difference in the integrity of the intestinal epithelial barrier between the two sexes of murine, following LPS-induced inflammation. The integrity of the epithelial barrier is regulated by tight junction proteins which aid in maintaining the structure and permeability. This study focuses on a member of the Claudin protein family, Claudin-3 (Cldn-3), and whether its absorption is sex-dependent. From the murine model, sections of the jejunum were analyzed 24 hours post-LPS treatment and quantified using NIH ImageJ in a double-blinded study.

Acknowledgments

I would like to thank Dr. Monica Carson and the Carson lab for mentoring me on this research project. I specifically thank Dr. Carson for being my faculty mentor and providing me with an exciting and innovative research experience as an undergraduate at UC Riverside. I also thank Ph.D. candidate Paula Da Silva Frost for allowing me to participate in her research. The guidance and experiences that I received shaped who I am as a scientist and allowed me to refine my research techniques. The skills I acquired will carry over into my professional career and I am grateful for the support I received.

I also extend my gratitude to University Honors for allowing me to participate in research with their support and guidance. The support that I received allowed me to excel in my undergraduate degree and explore different opportunities.

Table of Contents

Introduction

Physiological studies conducted by others in the Carson lab indicated that there was a change in intestinal epithelial integrity and that the integrity was differentiated by sex. Here we quantify one tight junction, Claudin-3 (Cldn-3), as another measure of an inflammatory response. This allowed us to investigate whether there was truly a difference in how the tissue responded to inflammation in male vs. female murine. The small intestine is an organ in the gastrointestinal (GI) tract that is made up of three parts: duodenum, ileum, and jejunum. The focus of this research was on the jejunum, due to it having the most robust response to the LPS injection. The jejunum is where most of the digestion and nutrient absorption takes place and large fluxes of water and electrolytes occur to make the contents of this facet of the small intestine isosmotic (Creamer). The jejunum can be seen below:
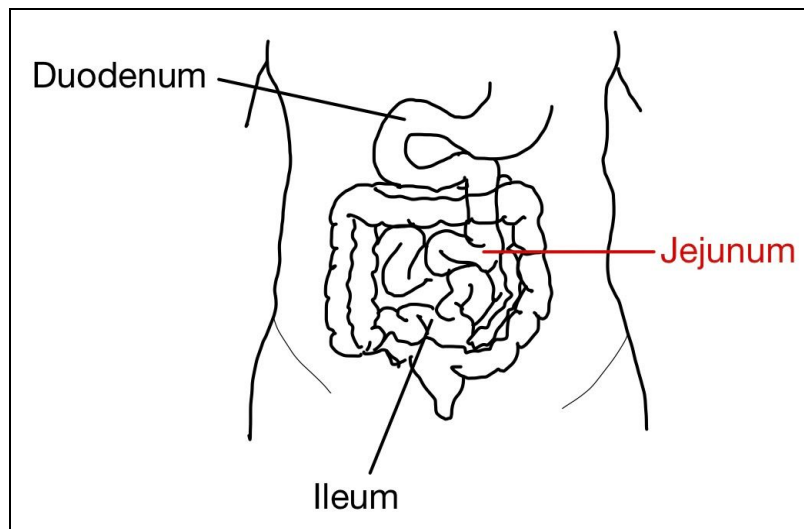

Figure 1. The small intestine is seen with its three facets: the duodenum, ileum, and jejunum. The jejunum is the primary focus of this research; thus, it is denoted in red.

Lipopolysaccharide (LPS) was injected into the peritoneal cavity in which the small intestine is located. LPS is a glycolipid component that is part of the bacterial wall of gram-negative bacteria (Bertani). Because LPS is a component of all gram-negative bacteria, the mammalian immune system has a dedicated array of receptors that detect the presence of LPS in the local environment. By binding immune receptors such as TLR4, LPS triggers a robust pro-inflammatory response in multiple immune cells which in turn regulates the function of tissues they are located in. Intraperitoneal injection results in a systemic body-wide inflammatory response. Thus, our experimental system models the body's response to bacterial infection within the peritoneal cavity. Here, we injected mice with LPS, and 24-hours post-injection, we quantified the response of the intestinal epithelium to the inflammatory trigger. We quantified both gene expression changes of the tissue by nanostring analysis and protein responses in histologic tissue sections.

In order to investigate the inflammatory response spatially, we quantified the internalization of Cldn-3 for two reasons. First, it is a tight junction protein that regulates epithelial barrier integrity. Second, its internalization is a reflection of the magnitude of the epithelial response to inflammatory triggers. Specifically, Claudin-3 is a member of the Claudin family, which plays an important role in regulating epithelial paracellular permeability (Ravi). The function of the tight junction can be seen in Figure 2. The Claudin family resides in tight junction strands and regulates barrier properties by controlling the charge and size properties of the paracellular space (Garcia). In the murine model, Cldn-3 mRNA is one of the most highly expressed in the GI tract out of the other Claudin proteins and it is easily visualized in histologic sections at the borders of cells in the healthy non-inflamed intestinal epithelia of the jejunum. In

our experiments, Cldn-3 was visualized by immunofluorescence in histologic tissue samples of the male and female murine jejunum to determine how much was internalized.
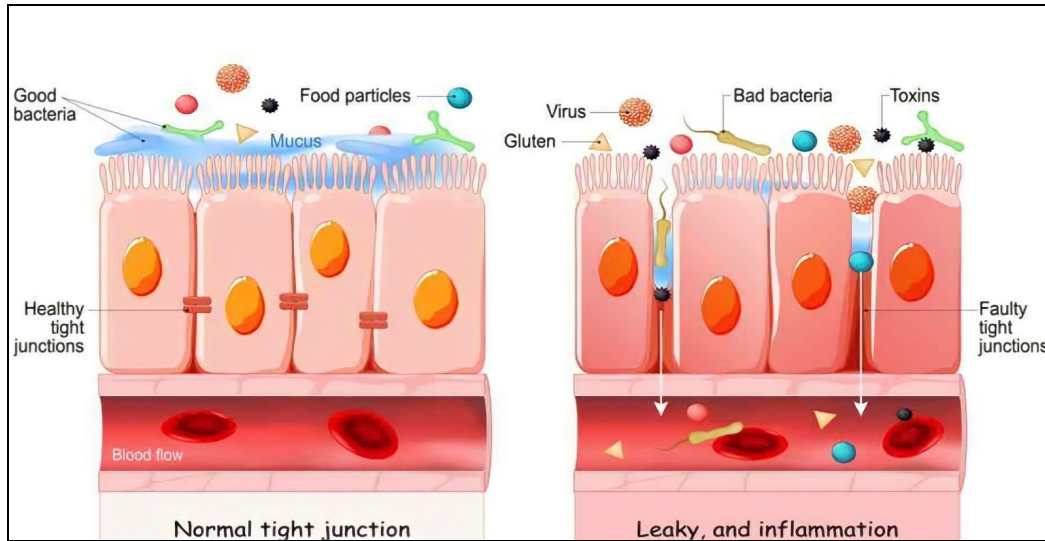

Figure 2. *The Role of Microbiota and Intestinal Permeability in the Pathophysiology of Autoimmune and Neuroimmune Processes.* The function of the tight junctions is demonstrated by the figure. Tight junctions hold together the intestinal cells. The focus of this research is on the tight junction Claudin-3.

Inflammation in the small intestine can lead to numerous conditions, including neurodegenerative ones, making it an important topic to investigate. The state of this organ can have destructive effects on the activity of the central nervous system, through its regulation of intestinal barrier function. The small intestine, an organ in the gastrointestinal tract, contains microbiota that has been linked to Alzheimer's disease and Parkinson's disease. Alzheimer's Disease is a progressive neurologic disorder that causes the brain to atrophy and deteriorate. Parkinson's Disease is a progressive disorder of the nervous system that affects mobility. The

connection between the gut microbiota—the set of microorganisms that reside in the GI tract—and the brain are of growing interest in recent years. Several studies have shown that the intestinal microbiota sends and receives signals to and from the brain to monitor numerous Gastrointestinal functions. This signaling is proven to play a role in brain development and function (Ceppa).

More specifically, inflammation in the small intestine itself can lead to a defect in the epithelial barrier. This defect has been observed in various intestinal disorders, such as Ulcerative Colitis, Crohn's disease, and Celiac diseases (Lu). Ulcerative Colitis occurs when the bowel is inflamed and causes ulcers in the digestive tract. Crohn's Disease can cause inflammation as well and results in extreme discomfort. Celiac Disease is when the ingestion of gluten can lead to damage to the gut. All three of these diseases occur when the small intestine is inflamed; therefore, it is an important issue to research. Inflammation in the jejunum could cause a chain reaction that would disrupt the homeostasis of the functioning body. Thus, determining whether there is a difference in inflammation in male vs. female murine can be used to further investigate the links between various diseases in other organisms.

Methods and Materials

Tissue Analysis:

For this experiment, both female and male naive murine were used. The two sexes were used to determine whether the response to LPS-induced inflammation is differentiated by sex. The tissue was analyzed after inflammation was induced via intraperitoneal injections of lipopolysaccharide (IP-LPS) for 24 hours. The jejunum is the primary focus because it yielded the richest response to the IP-LPS. The tissue analysis was conducted in a double-blinded methodology to ensure there was no bias to falsify the study. The images were then analyzed using NIH ImageJ. ImageJ is an open-source program designed to process multidimensional scientific images. The images were set at a distance in pixels of 11.387, in order to scale the image on ImageJ. Next, the images were converted to a green color and their brightness and contrasts were changed to increase the visibility of the Cldn-3. The images are of the villi of the small intestine, which are tiny projections into the intestinal cavity that increase the surface area for absorption.

Tissue Quantification:

The images were analyzed to search for little green dots, called puncta, within cells. These puncta indicate the presence of Cldn-3. The cells were classified as either apical incomplete (AI) or apical complete (AC), with the latter having a cell with complete borders. This classification was used to determine whether the puncta are internal to the microvilli (AC) or on the surface of the microvilli (AI). By including the cellular location in the analysis, we can test whether the inflammatory response of the jejunum is spatially regulated as well as sex-dependent. The parameters for measuring puncta were $0.065 < x$ area for the minimum size of

small puncta and x<0.291 area for the minimum size of large puncta. An additional parameter was that each sample needed at least five cells to be considered a good sampling. The cells were then quantified in an alphabetical order to ensure organization, with the puncta being quantified numerically. Also, viable cells that did not have a punctum present were denoted with an "X". We quantified the response of the jejunum tissue to the systemic LPS induced inflammation. First, we looked at the internalization of the Cldn-3 puncta. The differential response of Cldn-3 was quantified.

Statistical Analysis:

After all the images of the tissue were quantified, the average number of small and large puncta was calculated for each cell. This was conducted by counting the number of puncta in each cell, finding the total number of small and large puncta per cell, and averaging those values. In addition, the averages for apical incomplete and apical complete cells were calculated from the tissue samples. These calculations were conducted to organize the data for further analysis. After the data from the images was finalized, it was compiled using PRISM software. PRISM combines scientific graphing, statistics, and data organization. This software was used to graph the inflammation of the jejunum in female and male murine models. Using this software, we were able to determine if there was a statistical difference between the inflammation of male and female murine after treatment with LPS. The results were, thus, unblinded and the data was compared to determine whether the internalization of Cldn-3 was sex-differentiated. The blinded analysis from the team was unblinded and the team's data was pooled. The data was then used to determine if any trends were present in the data collected.

Table 1A-B. Data table created from the tissue sample in Figure 4 and Figure 5A-B. Table 1A is the data table of the puncta's cell location and size information. Table 1B is the statistical analysis of the tissue sample.

Puncta	Area	Mean	Min	Max	Cell Letter	Apical Incomplete?	AI frequency	Positive	Negative	Total Cells
1	0.093	167.833	150	190	A	y	3/7 y	7	9	16
2	0.093	171.583	152	191	A	y	4/7 n			
3	0.37	149.143	135	165	B	y	3 y: 4 n			
4	0.093	158.417	142	181	C	y				
5	0.093	162.333	142	175	C	y				
6	0.162	164.905	144	180	D	n				
7	0.069	184.333	168	200	E	n				
8	0.062	170.375	153	184	E	n				
9	0.069	161.667	131	178	F	n				
10	0.093	149	133	165	G	n				
11	0.123	159.375	145	168	G	n				
12	0.093	160.333	149	175	G	n				

Table 1A

Cell	Small	Large	Total
A	2	0	2
B	0	1	1
C	2	0	2
D	1	0	1
E	2	0	2
F	1	0	1
G	1	0	1
Total	1.285714286	1	1.428571429
AI	0.25	0	0.25
Complete	0.75	1	1.75

Table 1B

Gene Expression:

After the differential response of Cldn-3 was quantified, we next tested the response of the tissue by looking at gene expression. Gene expression is essentially the process by which information stored in our DNA is converted into a functional product, such as a protein. The process starts from DNA is converted to RNA by transcription, then translated into a protein. The process can be seen below in Figure 3.
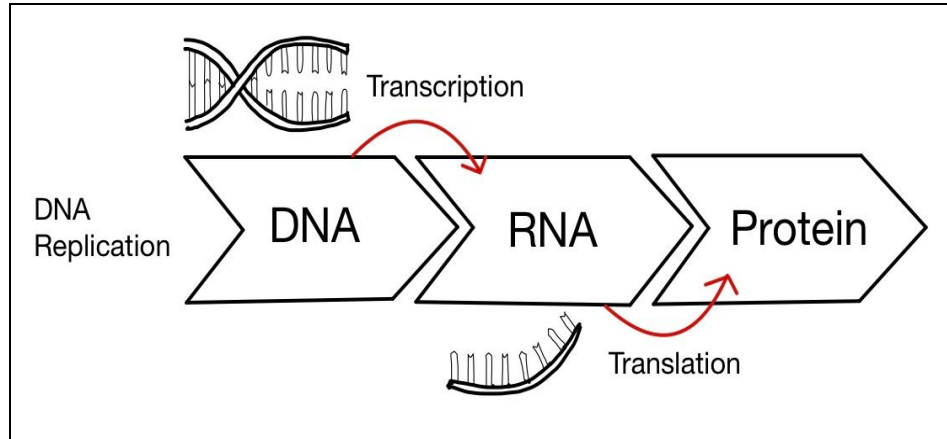
Figure 3. The process of gene expression is illustrated. DNA replication occurs and that DNA is converted into RNA via transcription and finally translated into protein. Nanostring utilizes this process in order to analyze the data.

The gene expression process was conducted by Nanostring hybridization counts. Nanostring is an amplification-free technology that counts molecules directly by measuring the content of nucleic acids. Gene expression analysis was performed by the graduate students in the lab by isolating the RNA from the jejunum in both male and female murine, following systemic LPS induced inflammation. Gene expression was done on a panel of inflammatory markers using Nanostring technology. After receiving the gene expression data, the data was analyzed and graphed. The graph can be seen in the Results section.

Results

While examining the jejunum of the murine small intestine, the Cldn-3 internalization
was observed. The green fluorescence allows the tissue sample to be visualized with each cell's
borders outlined. From this figure, further analysis was performed in order to quantify the puncta
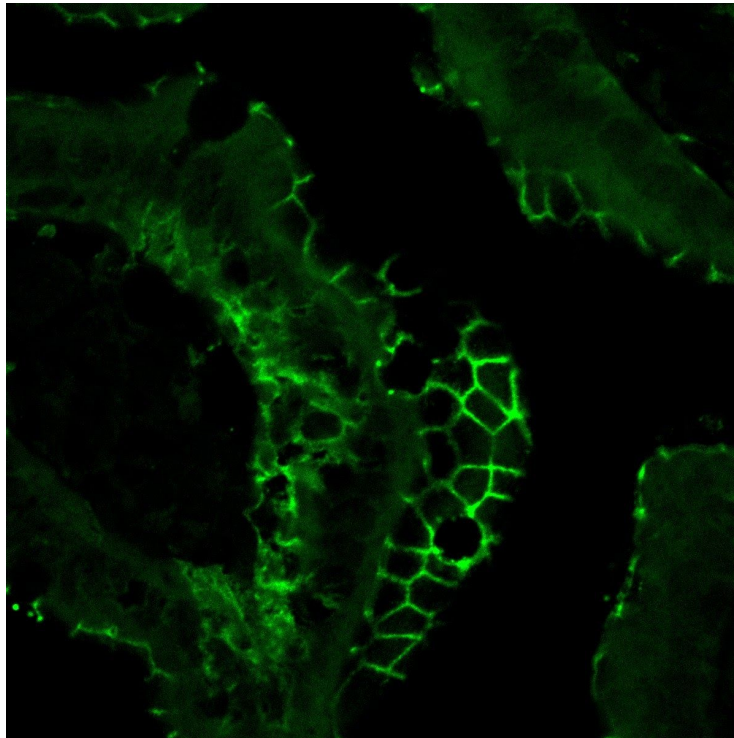using ImageJ.

Figure 4. Immunofluorescence stained tissue of Jejunum with the scale, color,
brightness/contrast altered to increase visibility (pre-quantification). The image was taken
under a microscope and uploaded to ImageJ for further analysis. Figure 4 is a low
magnification of the murine jejunum.

From Figure 4, puncta quantification took place to estimate the internalization in male and female murine. The figure below depicts a quantified image that followed the parameters previously mentioned.
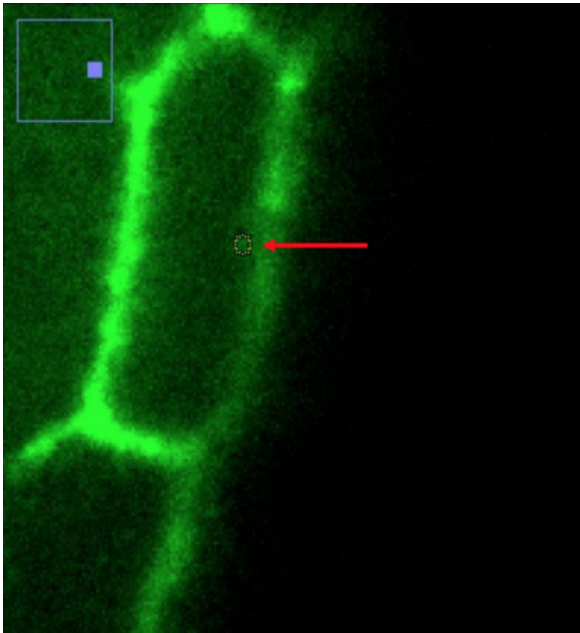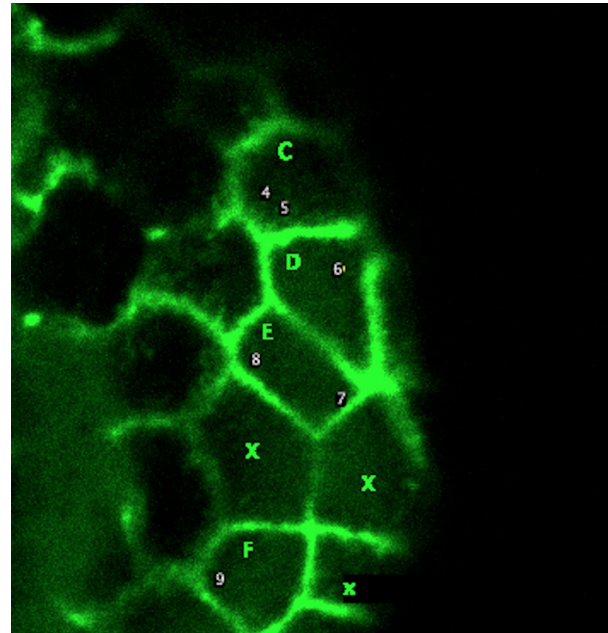

Figure 5A

Figure 5B

Figure 5A-B. Immunofluorescent image post quantification with the cells labeled alphabetically and puncta labeled numerically. Cells C and D exemplify an apical incomplete cell where a border is missing, while cells D and F exemplify an apical complete cell with four borders. The cells that are viable, but without puncta, are labeled with an "X". Figure 5A represents the puncta as indicated by the red arrow, while Figure 5B is the completed quantification of the image. Figure 5 is a high magnification of the murine jejunum.

Following puncta quantification, gene expression analysis was performed. The data from the male and female murine were combined to create a graph representing both of the sexes. The graph was created using gene expression technology, Nanostring, to observe patterns of how the

different pathways in the murine internalized Cldn-3 in response to systemic LPS induced

inflammation. The graph demonstrates that females showed a greater gene expression response

to LPS induced inflammation than males. The graph seen below helps answer the research

question: Is LPS induced inflammation sex-differentiated? As seen by the graph, it is

sex-differentiated as females and male murine responded differently. In the graph, the females

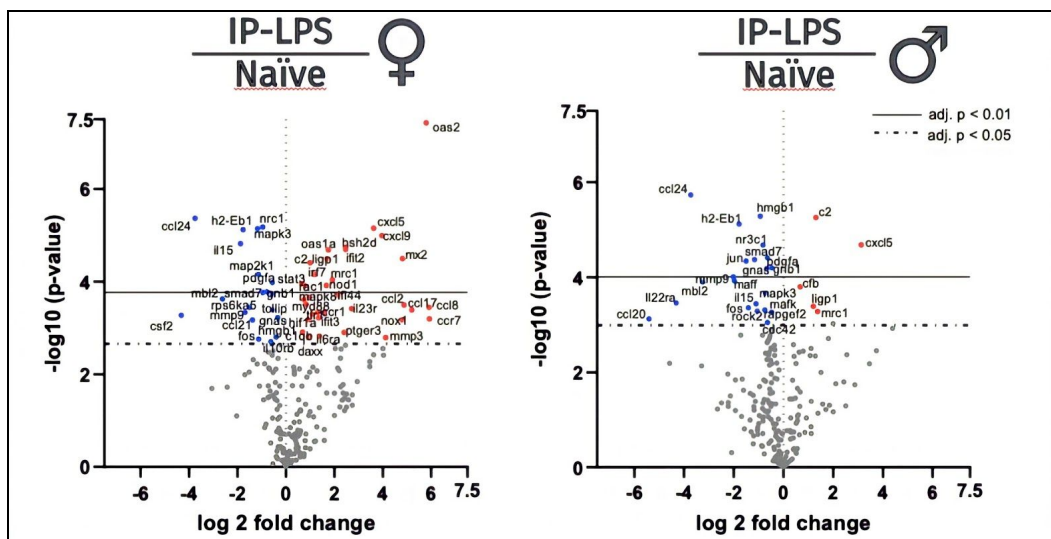have more red dots, corresponding to more upregulated genes.

Figure 6. The nanostring data shows upregulation of a greater number of genes in females than

in males. The analysis of male and female murine's differentially expressed genes in response to

LPS is observed. The red dots represent upregulated genes while the blue dots represent

downregulated genes.

Next, the different pathways of the murine were analyzed. An example is the fatty acid

oxidation pathway. This process takes place in the mitochondria and breaks down a fatty acid

into acetyl-CoA. This pathway was used to investigate whether the male or female murine

portrayed a different response, and if so, how significant was the difference. The following data

table demonstrates that there is a difference between the inflammation response in female and male murine. The wild-type murine are those that have a phenotype that appears in nature. In other words, they have not been experimentally altered in any way and serve as a control for the experiment. These results are shown by the table below:

Table 2. Data table of Wild Type Female with (+LPS) and without vs. Wild Type Male with (+LPS) and without.

Female		Male	
WT	WT +LPS	WT	WT +LPS
Y	Y	Y	Y
1.810	1.141	-1.666	-1.882
1.611	1.675	-2.313	0.060
1.763	1.234	-2.787	-0.645

Using the data from Table 2, PRISM was used to create graphs to visually represent the data. The graphs were used to visualize whether or not there was a difference between the male and female responses to systemic LPS induced inflammation.
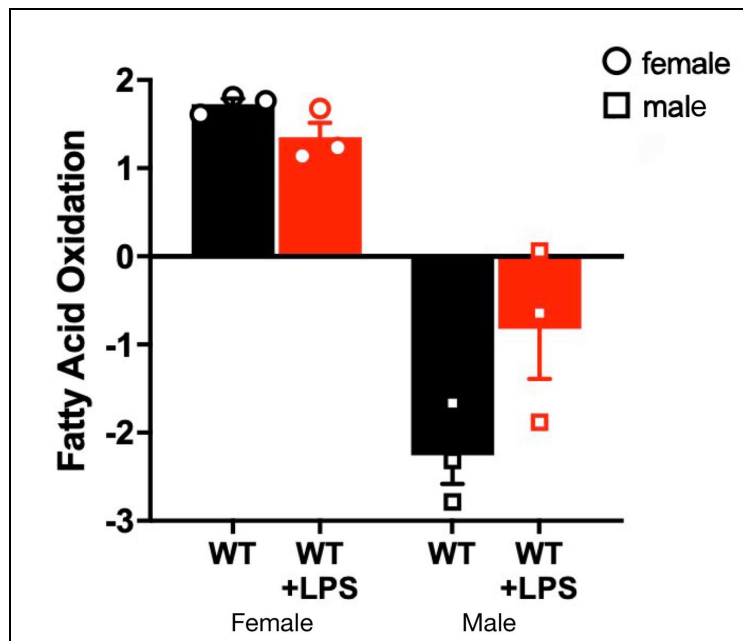
Figure 7: Corresponding PRISM graph from the data in Table 2. Male and female murine are differentiated by squares and circles, respectively. The effect of LPS injection on the fatty acid oxidation pathway is seen. This graph was based on the gene expression of the pathway.

At baseline, comparing the black bar with the circles (females) and the black bar with the squares (males), there is about a four-fold difference in the level of expression. There was no significant decrease in the fatty acid oxidation pathway after inflammation in females; however, the male baseline was about four-fold lower, and that in response to LPS it increased. Thus, in figure 7, the responses of males and females are very different, even in the wild type. The fatty acid oxidation is, therefore, lower in males than females. Females have greater upregulation and downregulation of more inflammatory genes in response to inflammation than males do. From the data in Table 2 and its corresponding graph in Figure 7, we conclude that the baseline homeostatic state of male and female jejunum are different. We also conclude that the females did not show a significant change, while the males showed an increase in fatty acid oxidation. However, even after this increase, the males were still nearly three-fold lower than the females. Therefore, LPS inflammation is determined to be sex-dependent by the results of puncta quantification, Nanostring technology, and PRISM graphing.

Discussion

The jejunum portrayed a strong inflammatory response in the fatty acid oxidation category; therefore, the function was affected by the inflammation caused by LPS. The jejunum as a structure functions in absorption, so how much does inflammation affect that physiological function? According to the literature, inflammation can be linked to various diseases, including neurodegenerative ones. Inflammation does not allow the jejunum to function as well as when it is not inflamed. This disrupts the homeostasis of the body, as nutrients are not being absorbed at the rate they usually are. The investigation of the link between inflammation and diseases of the central nervous system is highly popularized in recent years. Therefore, female murine demonstrated a greater upregulation and downregulation of more inflammatory genes in response to the LPS treatment than male murine do.

The use of Nanostring was efficient in this study because we had no gene amplification and were able to do direct quantification of transcripts. In addition, PRISM was also helpful in visualizing the data and determining if any trends could be seen. Both programming systems were easy to utilize and allowed for the data to be visualized and analyzed. The trend observed is that male and female murine displayed sex-differentiated effects of inflammation in the various pathways observed. This result was not surprising, as the wild types displayed sex-differentiated results as well. However, the extent of the difference was surprising. Females consistently displayed a more robust response to systemic inflammation than males. The Cld-3 data showed an increased internalization in LPS injected female mice than the wild-type mice, but this did not occur in males.

Conclusion

Furthermore, through investigating the jejunum of male and female murine after LPS treatment, it was concluded that the response to inflammation was sex-dependent. Figure 6 showed that females had a more robust response to the LPS induced inflammation. Females had a greater number of upregulated genes than males did. In both the wild-type and experimentally manipulated murine, females had different baseline homeostatic responses. Through puncta quantification, we were able to determine how much Cldn-3 was internalized. Following this, the data were statistically analyzed to determine if there were major differences. To visualize these differences, PRISM and Nanostring were utilized. PRISM and Nanostring helped reinforce the hypothesis that response to LPS inflammation is different in male and female murine. Therefore, the hypothesis was correct and the jejunum of male and female murine demonstrated different responses to systemic LPS induced inflammation.

Works Cited

Bertani, Blake, and Natividad Ruiz. "Function and Biogenesis of Lipopolysaccharides." *EcoSal Plus* vol. 8,1 (2018): 10.1128/ecosalplus.ESP-0001-2018. doi:10.1128/ecosalplus.ESP-0001-2018

Ceppa, Florencia Andrea et al. "Human Gut-Microbiota Interaction in Neurodegenerative Disorders and Current Engineered Tools for Its Modeling." *Frontiers in cellular and infection microbiology* vol. 10 297. 7 Jul. 2020, doi:10.3389/fcimb.2020.00297

Creamer, Brian. *The Small Intestine*. London: Heinemann Medical, 1974. Print.

Garcia-Hernandez, Vicky et al. "Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation." *Annals of the New York Academy of Sciences* vol. 1397,1 (2017): 66-79. doi:10.1111/nyas.13360

Lu, Zhe et al. "Claudins in intestines: Distribution and functional significance in health and diseases." *Tissue barriers* vol. 1,3 (2013): e24978. doi:10.4161/tisb.24978

Morris G, Berk M, Carvalho AF, Caso JR, Sanz Y, Maes M. *The Role of Microbiota and Intestinal Permeability in the Pathophysiology of Autoimmune and Neuroimmune Processes.*

Ravi M. Patel, Loren S. Myers, Ashish R. Kurundkar, Akhil Maheshwari, Asma Nusrat, Patricia W. Lin, "Probiotic Bacteria Induce Maturation of Intestinal Claudin 3 Expression and Barrier Function." *The American Journal of Pathology*, vol 180, 2 (2012): Pages 626-635, ISSN 0002-9440, https://doi.org/10.1016/j.ajpath.2011.10.025.