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Effects of Propionate on Macrophage Migration

Honors Thesis

Cian James Callahan

Department: Biology

Advisor: Yvonne Sun, Ph.D.

April 2023

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Abstract

Macrophages are leukocytes that play an important role in the antibacterial responses by our body's immune system. The activities and functions of macrophages are influenced by a variety of substances, such as short chain fatty acids (SCFAs) found in the gut. Currently, we know that SCFAs, such as propionate, induce directional recruitment of leukocytes. For intracellular bacterial pathogens, the movement of infected macrophages can contribute to the systemic dissemination of the pathogens. However, little is known whether SCFAs like propionate can modulate the movement of infected macrophages. To fill this knowledge gap, *Listeria monocytogenes*, a human pathogen capable of causing infections with high mortality rates, is used as the model intracellular pathogen. It is not clear how propionate modulates activities of infected macrophages. The first objective of my honors thesis is to develop a transwell protocol to assess macrophage migration, including the identification of optimal staining procedures, macrophage numbers, and transwell pore sizes. The second objective of my honors thesis is to investigate how propionate changes the migration of infected macrophages. Findings from this study can help us better understand regulatory signals for macrophage functions and reveal potential immunotherapeutic treatments against intracellular infections.

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Introduction

Macrophages as phagosomes

Macrophages are an important type of white blood cell, or leukocyte, that can eliminate a variety of intracellular pathogens. Differentiated macrophages are found in a variety of tissues and have a specific function for each particular tissue. Alternatively, monocytes, which are precursors of macrophages, can also be recruited to tissues in the presence of infection. (21.2 Barrier Defenses) At the site of infection, macrophages can eliminate pathogens through the process of phagocytosis after recognizing specific signals on the invading pathogens. These signals, collectively known as pathogen associated molecular patterns (PAMPs), include peptidoglycan found on the cell walls of bacteria, lipopeptides, and DNA or RNA. These PAMPs are detected through pattern recognition receptors (PRRs), which include surface and intracellular proteins used by macrophages to detect foreign pathogens. For example, Toll-like receptor 5 (TLR5) recognizes bacterial flagellin while TLR4 recognizes bacterial lipopolysaccharides (LPS). After the binding of PAMPs and PRRs, macrophages are transformed from a naive state to an activated state. Activated macrophages can produce pro-inflammatory cytokines, such as IFN-γ, and TNF$\boldsymbol{\alpha}$, to stimulate additional antimicrobial processes to degrade pathogens. (Alberts)

Phagocytosis is a multi-step process where extracellular particles are engulfed into an intracellular phagosome and subsequently degraded as the phagosomes undergo maturation. The process of phagocytosis begins when macrophages form a pseudopod to engulf the pathogen into a membrane compartment called phagosome. The phagosome then matures by fusing with other vesicles in the cytoplasm to create an acidic and

degradative compartment. The maturation and acidification of the phagosome can be identified through specific markers. The early phagosome, at a pH of roughly 6.5, contains markers such as Rab5, EEA1, and PI3P proteins. (Günter) Rab5 is a type of GTPase protein that initially regulates the phagosome and plays a role in lysosome fusion and degradation of pathogens. (Alvarez-Dominguez) The late phagosome, at a pH of roughly 5.5, contains Rab7, PI3P, and LAMP-1 markers. LAMP-1 proteins serve as a ligand to bind the phagosome with lysosome, creating the phagolysosome that has a pH of approximately 4.5. The increased acidification of the phagolysosome activates pH-dependent enzymes to produce reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, to degrade the particles inside the phagolysosome. The production of ROS also requires an increased uptake of oxygen, resulting in an overall lower oxygen availability. Once degradation of the pathogen is complete, waste products are excreted from the cell through exocytosis. (17.3 Cellular Defenses; Günter)

Macrophages in the gut and chemotaxis

The gastrointestinal system is heavily exposed to foreign pathogens and is thus the largest compartment of the immune system. (Bain) Resident immune cells in the gut include mononuclear phagocytes (MPs), such as macrophages and conventional dendritic cells, as well as derived monocytes that respond to pathogen recognition. (Watanabe) Resident and derived gut macrophages play an important role in tissue injury and infection for various reasons, one being their release of chemokines.

Chemokines are small proteins of roughly 60-100 amino acids and are secreted by immune cells, such as macrophages, as well as tissue cells, in response to proinflammatory chemokines. They play an important role in the immune system by regulating cell trafficking toward sites of infection or injury. For example, when a tissue has been infected, resident macrophages and other cells will secrete chemokines, which recruit monocytes and other immune cells to the source of chemokine secretion, moving along a concentration gradient. (Deshmane). Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, is a member of the C-C chemokine family, which refers to it having two adjacent cysteines. MCP-1 is a potent chemotactic attractor for monocytes and is produced by a variety of cells, such as tissue and immune cells.

Macrophages have two forms of movement toward chemokines: amoeboid and mesenchymal. Amoeboid migration is independent of integrin, an adhesive molecule, and is characterized by flowing and squeezing. This is the most basic form of cell movement. Mesenchymal migration requires an adhesive molecule and is characterized by the leading edge adhering to a surface while the lagging edge detaches and retracts ahead. The density of adhesive molecules on a surface is a regulator of mesenchymal migration; too many adhesive molecules and the cell cannot detach, while too few adhesive molecules the bond is not supported. (Cui)

How propionate effects macrophage function

Macrophage recruitment and phagocytosis are highly controlled processes that can be regulated by various environmental signals. For example, pro-inflammatory cytokines can upregulate phagocytosis in macrophages. (17.3 Cellular Defenses) Other

chemical products, such as propionate, also exhibit regulatory activities on macrophage function. Propionate is a short-chain fatty acid (SCFA) derived from the breakdown of dietary fibers and the fermentation of non-digestible carbohydrates by our gut microbes using various metabolic pathways. The resulting SCFA concentrations in mammals were reported to range from 10 to 100 mM in the colon and 0.1 to 10 mM in the bloodstream. (Venegas) Multiple studies have shown that SCFA at low levels can strongly affect macrophage functions, including reducing the production of pro-inflammatory cytokines (Vinolo, Bloes, Vinolo2, Sina), regulating macrophage population (Shaw), inhibiting stimuli-induced expression of adhesion molecules (thereby slowing mesenchymal migration) (Vinolo), restricting viability of iNOS, and enhancing the production of IL-10. (Venegas) This suggests that propionate has anti-inflammatory regulatory functions.

Although it has been shown that propionate affects macrophage recruitment and maintenance, it is not clear how propionate modulates activities of infected macrophages. For intracellular bacterial pathogens, the movement of infected macrophages can contribute to the systemic dissemination of the pathogens. For example, *Salmonella enterica* are resistant to certain macrophage antimicrobial properties and can grow within the macrophage. Steele found that *S. enterica* can be transferred from infected macrophages to healthy macrophages through contact dependent mechanisms, thus evading immune responses triggered by leaving the macrophage. (Sina)

Therefore, the first objective of my honors thesis is to develop a transwell protocol to assess macrophage migration, including the identification of optimal staining procedures, macrophage numbers, and transwell pore sizes. The second objective of my

honors thesis is to investigate how propionate changes the migration of infected macrophages.

Methods:

Macrophage culturing techniques:

RAW 264.7 murine macrophages (ATCC, TIB-71) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, SH30284.01) media containing 11% fetal bovine serum (FBS) (HyClone, SH30910.03) and 1% penicillin/streptomycin (Corning Cellgro, 30-002-Cl). Cells were incubated in a tissue culture dish at 37.0°C and 5% CO_2, until cells reached ~90% confluence. At this point, cells were scraped from plate, pelleted at 1500 RPM for 3 minutes at 21.0°C, and resuspended in fresh FBS DMEM. Macrophages were discarded 30 days after thawing.

Optimizing macrophage migration protocol:

Migration assays were performed using 8 μm inserts (Fisher, 07-000-396) in a 24 well plate (WWMP, 50051530) (Yang). RAW 264.2 macrophages were resuspended in serum free DMEM at three different concentrations (cells/well): 2.5 x 10^4, 2.5 x 10^5, 2.5 x 10^6 (Figure 1). Then, 100 uL RAW macrophages were added to well inserts, while 600 uL serum free DMEM was added to the basolateral compartment of the 24 well plate (Figure 2). Cells were allowed to migrate for 1, 3, and 24 hours in an incubator at 37.0°C and 5% CO_2. These concentrations and times were chosen based on previous experiments (Justus, Yang, Green, Yakubenko, Park, Carr). After allotted time, transwells were removed from serum free DMEM, dried using a cotton-tipped applicator, and fixed using

70% ethanol. After 10 minutes, cells were removed from ethanol, dried, and stained using 0.2% crystal violet. Cells were then dipped into distilled water to remove excess CV and dried (Justus). Transwell membranes were visualized using an inverted microscope. Images were taken of each well and macrophages were counted. Concentration of macrophages in the basolateral region was counted using a hemocytometer. Similarly, the above protocol was repeated with 1 μm inserts (Fisher, 7000394) with 1 x 10^6 cells/well. As negative controls, the above protocol was repeated without macrophages to determine baseline and optimal crystal violet (BD, 2016-01-31) concentrations.

	8 um		
Time point 1 1 hours	2.5 x 10^4 cells/well	2.5 x 10^5 cells/well	2.5 x 10^6 cells/well
Time point 2 3 hours	2.5 x 10^4 cells/well	2.5 x 10^5 cells/well	2.5 x 10^6 cells/well
Time point 3 24 hours	2.5 x 10^4 cells/well	2.5 x 10^5 cells/well	2.5 x 10^6 cells/well

Figure 1 Transwell Experiment Setup

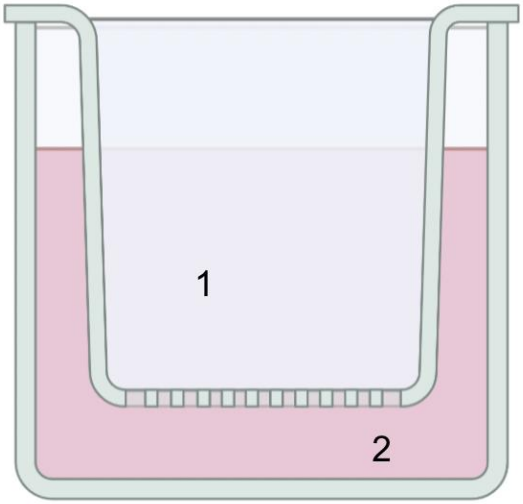

Figure 2: Visualization of A Transwell Macrophages were added to the inner transwell compartment (1), which was separated from the basolateral compartment (2) by a porous membrane.

Transwell protocol with MCP-1:

The migration assay protocol mentioned above was used with the following modifications. Only 1 μm transwells were used. MCP-1 was added using the following concentrations: 20 ng/mL and 50 ng/mL (citation) to serum free DMEM in the basolateral compartment. Macrophages were allowed to migrate for 1 and 3 hours in an incubator at 37.0°C and 5% CO_2. Fixing and staining protocols were performed as previously described. Imaging protocol was modified by using the Nikon Eclipse Ts2 Microscope in Dr. Loan Bui's lab. Four unique images were taken of each transwell, and the average macrophage concentration from the four images was calculated.

Transwell protocol with MCP-1 with propionate:

The migration assay protocol was used with the following modifications. Only 20 μg/ml of MCP-1 was used in this experiment. Twenty-four hours prior to beginning the migration assay, DMEM media was aspirated from three cell culture wells. Fresh media containing no propionate, 0.1 mM propionate, and 1.0 mM propionate (Fisher, A162-500) were added to three different cell culture plates. Macrophages were incubated overnight at 37.0°C and 5% CO_2. Cells were allowed to migrate for 3 hours in an incubator at the same conditions. Fixing, staining, and imaging protocols were performed as previously described.

Results:

Protocol Development:

To understand how macrophage migration is modulated by various signals, we first needed to develop a reliable and consistent protocol in the lab. Therefore, concentrations of cells and crystal violets, transwell membrane pore sizes, and incubation time were systematically tested to identify optimal settings. In collaboration with another honors thesis student, Hannah DeRespiris, we found that concentrations of 2.5×10^4 and 2.5×10^5 cells/well resulted in very few macrophages in the transwell after any period of time (Figure 3a, b). However, 2.5×10^6 cells/well provided ample macrophages on the stained transwell (Figure 3c). Furthermore, we found that 24 hours of migration did not result in a higher number of cells on the transwell membrane (Figure 3d). It is likely that

macrophages already passed through the membrane or died in the serum-free media. Therefore, we continued with 1 and 3 hours of migration in subsequent experiments.

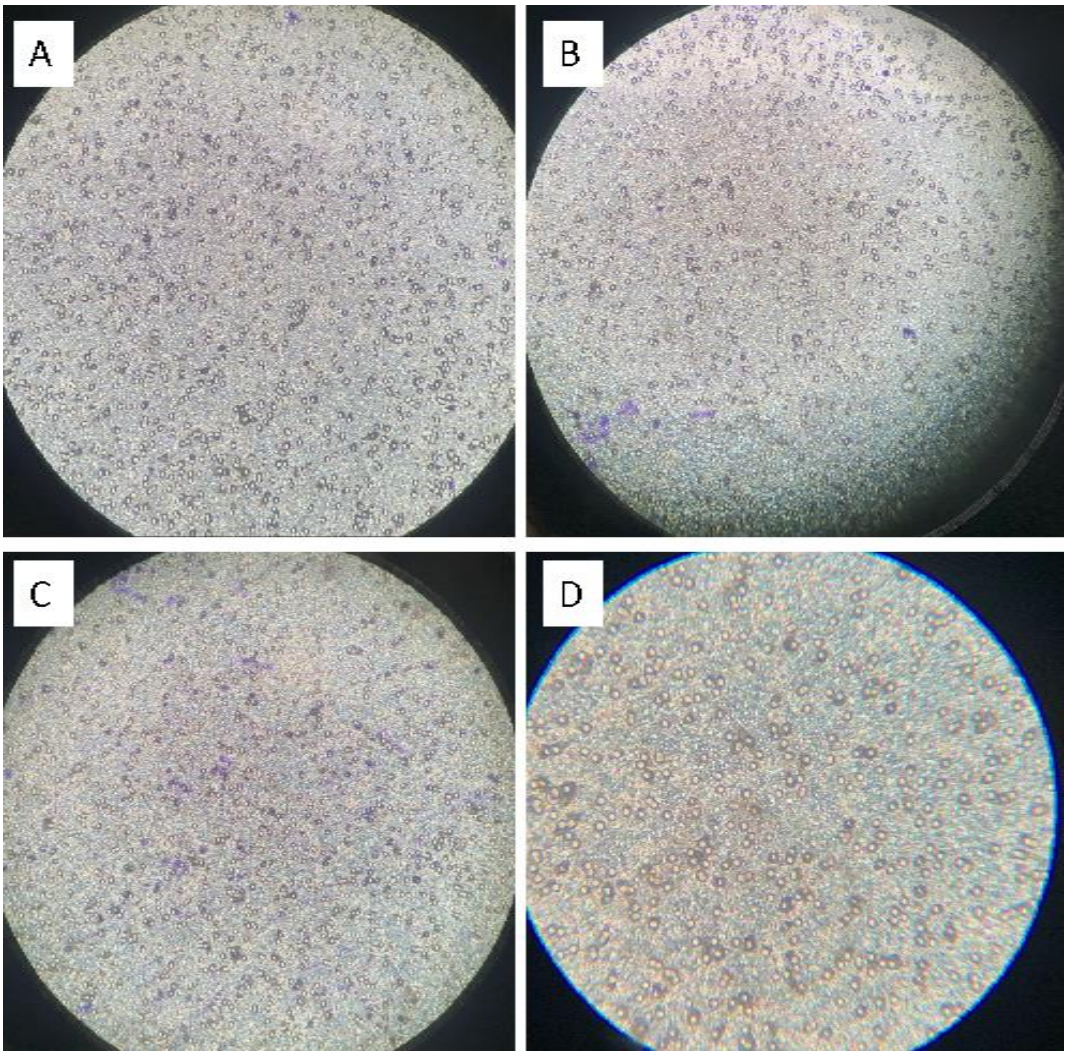

Figure 3: Transwell images with different macrophage concentrations. Images A, B, and C are stained transwells after 3 hours of migration. 2.5 x 10^4 cells/well of macrophages were added to image A, 2.5 x 10^5 cells/well to image B, and 2.5 x 10^6 cells/well to image B. Image D depicts 2.5 x 10^6 cells/well after 24 hours of migration.

To detect macrophages that have passed the transwell membrane, crystal violet (CV) was used to stain the cells. Two different CV concentrations (0.2 and 1.0% [wt/vol in ethanol]) were tested using similar migration assays. We found that 1% CV resulted in high background staining while 0.2% accurately stained transwells without overstaining (Figure 4).

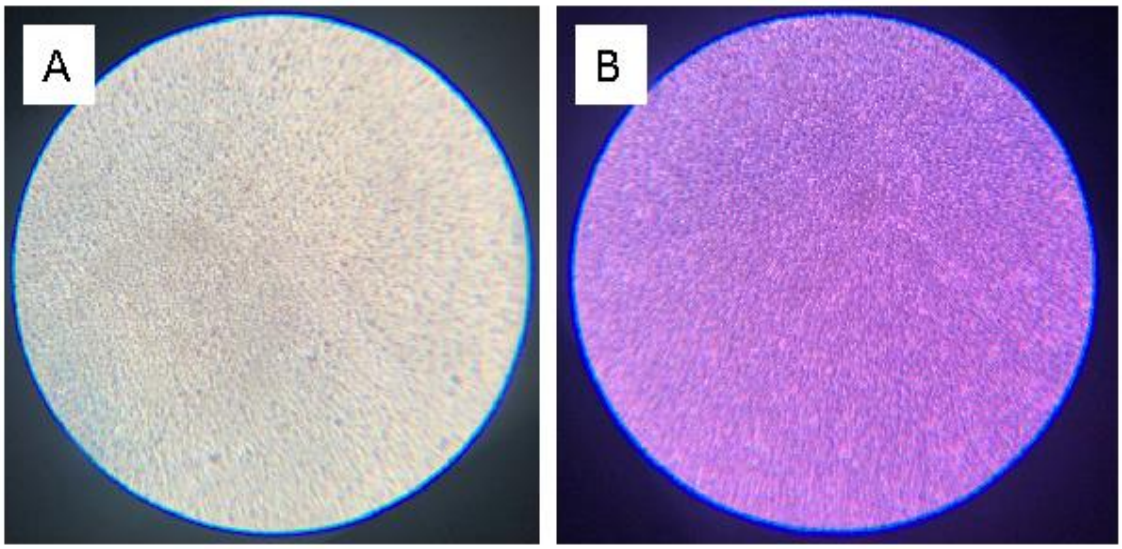

Figure 4: Transwell images with different CV concentrations. Image A was stained with 0.2% CV, while image B was stained with 1.0% CV in a 1 μm transwell.

Macrophages have the ability to move through membranes containing both 1 μm and 8 μm pores. To create a consistent protocol, we compared 1 μm and 8 μm inserts and found that macrophages could be seen on both. Therefore, either insert would be viable. We decided to proceed with 1 μm inserts as we did not have enough 8 μm inserts to continue our experiments.

Transwell protocol with MCP-1:

To determine optimal MCP-1 concentrations, 20 µg/mL and 50 µg/mL were compared. The following images are examples of stained transwells that were taken using Nikon Eclipse Ts2 Microscope in Dr. Loan Bui's lab. Stained macrophages (areas of dark purple) are found both isolated and bound to other macrophages. Results indicated that 20 µg/mL of MCP-1 was sufficient in allowing macrophages to migrate through the transwell. Further, 1 hour was no longer necessary as macrophage migration was too low at this time (Figure 5).

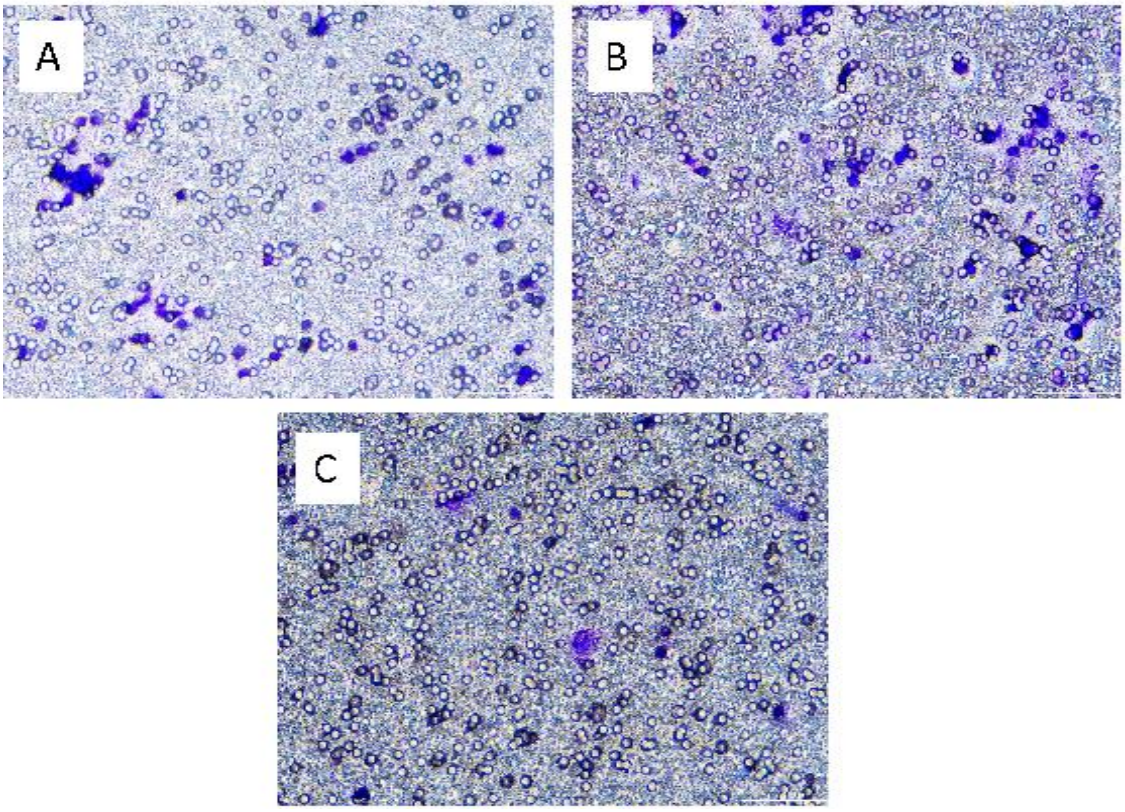

Figure 5: Transwell images after 3 hours of migration. No MCP-1 was added to image A, 20 µg/mL to image B, and 50 µg/mL to image C.

Transwell protocol with MCP-1 with propionate:

Macrophages were cultured in different concentrations of propionate for 24 hours to determine the effect of propionate on macrophage migration. The following images are examples of stained transwells and were taken using the Nikon Eclipse Ts2 Microscope in Dr. Loan Bui's lab (Figure 6). Stained transwells were counted and analyzed using Microsoft Excel. As shown in Table 1, no significant difference in cell number was seen between no propionate vs 0.1 mM propionate ($p=0.167$) as well as no propionate vs 1.0 mM propionate ($p=0.847$).

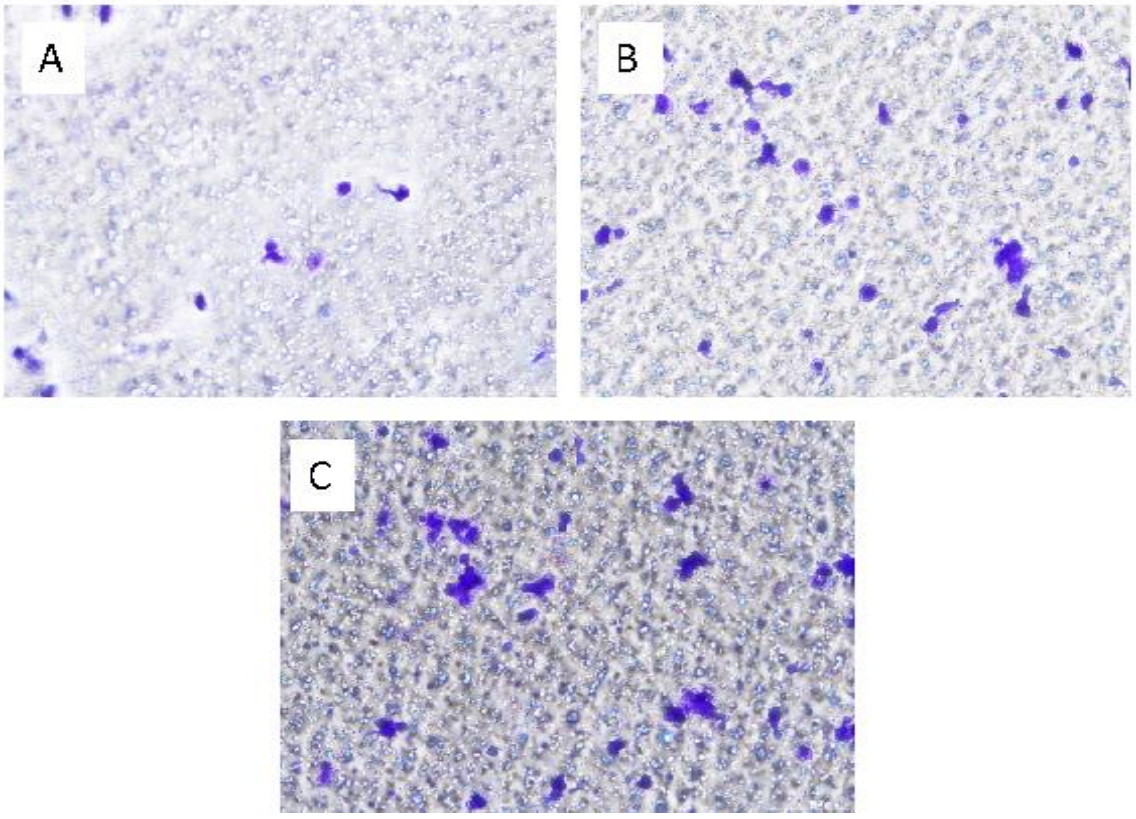

Figure 6: Transwell images after 3 hours of migration. Image A contains no propionate, image B contains 0.1 mM propionate, and image C contains 1.0 mM propionate.

	No Propionate		0.1 mM propionate		1.0 mM propionate	
3 hrs.	203	40	116	73	292	n/a
	240	159	9	9	195	129
	170	163	126	88	220	n/a
	30	21	74	62	52	40
	66	65	81	107	26	51
Total	**1157**		**745**		**1005**	
Average	**115.7**		**74.5**		**125.6**	
SEM	**25.214**		**12.647**		**32.269**	

Table 1: Each condition, no propionate, 0.1 mM propionate, and 1.0 mM propionate, had ten wells. Four images were taken of each well, and the total number of macrophages counted on each well is listed above. "**Total**" refers to the total number of macrophages counted as a sum of all the wells of that condition. Averages and standard deviation of the mean were calculated using Microsoft Excel. Errors occurred in two transwells which were unable to be counted and are labeled "n/a."

Conclusion and Reflection

In conclusion, my honors thesis has led to the creation and optimization of a macrophage migration protocol for the Sun lab. This protocol has been adequately documented and can be implemented in further studies for the lab. The second objective of my honors thesis, to determine the effects of propionate on infected macrophages, could not be reached due to time restraints. Future studies should include continuing to uncover and understand of propionate's effects on macrophage migration in the presence of MCP-1. This could lead to studying the effects of propionate on macrophages infected with *Listeria monocytogenes*, a pathogen which is heavily studied by the Sun lab. This

could lead us to better understanding the role of propionate in *L. monocytogenes* escape from the macrophage and systemic infection.

Throughout my honors thesis process, I have learned many important lessons that relate both to my future career as a researcher and to myself as an individual. There were many instances where I sat in Dr. Sun's office while she explained to me a complex pathway or a perspective of an idea that I had not yet considered (nor would have ever thought to consider). At first, I left these conversations feeling overwhelmed, frustrated, and exhausted. It is difficult for a recovering perfectionist such as myself to struggle understanding concepts that I have been studying for so long, or to go through an entire experiment to end with unwanted results. I think this is because in my normal coursework, we are given material and are tested on that material. This process is important as it is the foundation for creating deeper knowledge but is quite the opposite of research. In research, we use the foundation that has been created for us to try to explain a deeper concept that we do not fully understand. The challenge with this, which the researcher must strive to overcome, is that the explanation, or the question asked, or the hypothesis, is not always as is expected. In fact, the opposite is true, and the researcher is more often wrong in their hypothesis. What I have come to understand, and to love, about research is that wrong does not mean meaningless. Being wrong means that we have found one less way to answer our hypothesis. Being wrong means that we are one step closer to finding that answer.

Acknowledgements

I would like to first thank Dr. Yvonne Sun for her continued friendship and mentorship throughout my honors thesis process and beyond. Her ability to provide clear and concise instructions, as well as constructive criticism, has made me a better researcher and thinker. But beyond that, her open-mindedness and constant trust in me have impacted my academic and personal growth and allowed me to believe in myself and my abilities as a researcher. I am beyond grateful.

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