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Chasing Traps in Tiny Worms: Uncovering the Presence of Neutrophil Extracellular Traps in Caenorhabditis elegans

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Chasing Traps in Tiny Worms:
Uncovering the Presence of Neutrophil Extracellular Traps in *Caenorhabditis elegans*

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Preface and Acknowledgments:

As a freshman in college in the fall of 2019, pursuing a career in Medicine, I discovered that Regis University had various opportunities to do research in any of their science departments. At the time, I had little to no exposure to the different fields of science (microbiology, ecology, immunology, etc.) While looking for the best fit, I discovered the field of immunology. I had never realized how important our body's immune response was to keep us alive every day. Pathogens are everywhere, and our body is constantly fighting against them to keep us going. Surprisingly, a semester after I started getting my feet wet in this interesting world, Covid-19 happened. I am aware that this global pandemic has brought tragedy to many families in the world. However, it benefited me in some ways, since it helped me discover my professional goals. It motivated me to continue my research in immunology because I truly enjoy learning and understanding how we are designed to protect ourselves from threats like Covid-19, and how everyone has a different reaction to it. Inspired by this, I decided to start my research on a topic that has not yet been explored, how Neutrophil Extracellular Traps support the immune response in the nematode *C. elegans*, and how that knowledge has been translated to the human body to support the human's immune response against pathogens.

With that being said, I would like to thank all of those splendid characters in my college career that helped me to be where I am today. I would like to start with Dr. Taylor Jason Taylor and his wife, Mindy Taylor, for all of the support they gave me since day one. Dr. Sterling for her guidance and incredible support during my time at Regis University. Dr. Lucas for becoming another support system later in my college career and agreeing to research this unique topic. The Regis University Biology department, for sharing their work and laboratory tools with me to make this possible. To the honors program sponsors for encouraging me to record my research in

this paper that would be part of me for the rest of my life. Last but not least, to my parents for always giving me the freedom of pursuing my dreams.

Diego Carrasco **Biochemistry**

Chasing Traps in Tiny Worms: Uncovering the Presence of Neutrophil Extracellular Traps in Caenorhabditis elegans

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Abstract:

Since 2020, the immune system has become humankind's most powerful weapon to the challenges we have faced during the Covid-19 pandemic. Subsequently, scientists throughout the world have combined their brilliant minds to explore how spectacular our immune system is, and we have found new components of its mechanism(s) and pathways. Among those components, Neutrophil Extracellular Traps (NETs) stand out for their important contribution to humans' innate immune system. NETs have revolutionized the way scientists approach treatments for viral and bacterial infections. To understand NETs, I experimented with the nematode *Caenorhabditis elegans* to provide a better explanation and evidence on why these traps could become a key factor for modern medical treatments. A crucial topic covered in this thesis is the importance of the nematode *C. elegans* as a research model in medicine. I explain~~ed~~ the advantages of choosing this organism over others, and why it could be exploited in the research community to perform research studies. Some advantages are, its simplicity and genetic tractability make it an excellent organism for genetic studies, while its transparency allows for the visualization of cellular processes and signaling pathways. *C. elegans* also has a well-characterized nervous system and shares many genetic and physiological similarities with humans, making it a relevant model for studying human diseases and drug discovery.

Additionally, C. elegans is inexpensive and easy to maintain in the laboratory, making it a valuable tool for large-scale genetic and chemical screens. Overall, C. elegans has become a widely used model organism in various fields of research, contributing to our understanding of biological processes and disease mechanisms. NETs are important for providing a more personalized approach to medicine. In this research, I aimed to investigate the presence of NETs in the model organism C. elegans. Although the study was limited in scope due to time constraints, the findings presented in this thesis provide valuable information that can aid future researchers in this area of study. The study of NETs has emerged as an important area of research, and the information presented in this thesis may serve as a foundation for further investigations into the role of NETs in C. elegans and their potential implications for broader biological processes.

Chapter 1: Neutrophil Extracellular Traps, Immune Response

Neutrophils are innate immune phagocytes, part of the defense system which we are born with, that have a central role in immune defense (Papayannopoulous, 2018). Neutrophils are the most abundant phagocytes in the human bloodstream (Amulic, 2012). A phagocyte is a type of cell within the body capable of engulfing and absorbing bacteria and other small cells and particles (Glenn, 2019). The neutrophil plays a crucial role in the host's immune defense mechanism, acting as a sentinel against invading pathogens. It accomplishes this by releasing a sophisticated network of DNA, histones, and antimicrobial substances, which effectively neutralizes harmful microorganisms. These cells are most likely to be recruited from the blood to the site of infection early in the immune response (Lord, 2001).

Our understanding of the role of neutrophils in pathogen clearance, immune regulation, and disease pathology has advanced dramatically in recent years. Neutrophils engulf and kill bacteria when the antimicrobial granules fuse with the phagosome (Brinkmann et al., 2004). A phagosome is a tiny sac created by the inward folding of the cell's outer layer, along with its related lipids and proteins, during the process of phagocytosis, where it engulfs microbes or microbial proteins. Web-like chromatin structures known as neutrophil extracellular traps (NETs) have been at the forefront of this renewed interest in neutrophil biology (as shown in Figure 1). NETs were discovered not too long ago. NETs had their debut in an article based on a research study directed by Dr. Volker Brinkmann, a very well-known researcher that specializes in pathogenic infections back in 2004 (Brinkmann et al., 2004). Understanding the role of NETs in autoimmunity may facilitate the diagnosis of autoimmune diseases and the development of tailored therapies in the future (Lee et al., 2017).

Our immune system fights infections and illnesses. It prevents us from getting sick, or, if we do, it helps us get better. It's a system of different organs, cells, and proteins such as antibodies (Waldman, 2019). Together, the antibodies identify, attack, and destroy pathogens and other foreign substances. But sometimes the immune system makes a mistake and attacks part of the body. This is called autoimmunity. Thus, the identification of molecules that modulate the release of NETs has helped to refine our view of the role of NETs in immune protection, inflammatory and autoimmune diseases, and cancer (Papayannopoulous, 2018). The nuances of NET formation in various physiological contexts including under bloodstream, tissue, alkaline pH, and hypertonic conditions are just beginning to be explored (Ravindran, 2019).

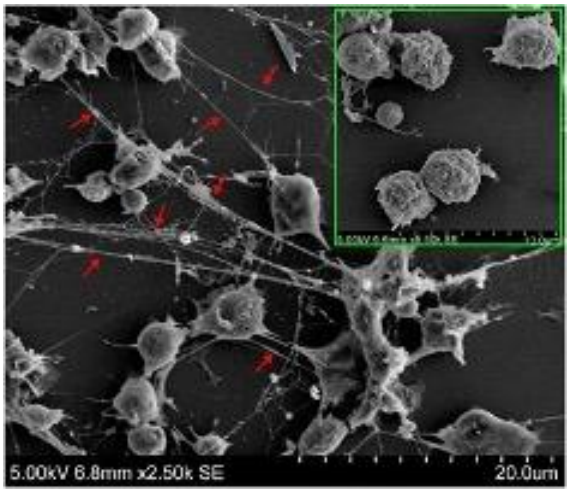


Figure 1. NETs trap pathogens in the bloodstream (red arrows). Figure from (Zhao, 2017).

To obtain accurate data on how effective (or not) NETs are in the immune system of living organisms, we decided to analyze them in *Caenorhabditis elegans*. *C. elegans* has been a valuable model organism for scientific research, and many important discoveries have been made using this nematode. Model organisms are essential in research for several reasons. One of the primary reasons is that they offer a simplified and controlled system to study complex biological

processes. These organisms are chosen for their ease of maintenance and manipulation, well-understood genetics and biology, and rapid growth and reproduction (Ankey, 2021). By using these model organisms, researchers can investigate the function of specific genes, proteins, and pathways in a living organism in a way that is not possible in more complex organisms, such as humans. Model organisms like fruit flies, mice, zebrafish, and nematodes are used to study the molecular basis of diseases and the effects of genetic mutations on the organism (Irion, 2022). Another significant advantage of using model organisms is that they provide a common framework for researchers to share knowledge and discoveries. Using the same model organism allows researchers to compare their findings and build on each other's work, leading to a more rapid accumulation of knowledge and understanding. This is particularly important in fields like genetics and developmental biology, where researchers use the same model organisms to study the underlying mechanisms that govern growth and development. In addition to being useful for basic research, model organisms can also serve as stand-ins for humans in certain contexts. For example, researchers use mice to test the safety and efficacy of new drugs and therapies before they are used in clinical trials. By doing so, they can identify potential side effects or risks and ensure that the treatments are safe and effective. Model organisms can also be used to study the effects of environmental factors, such as radiation or pollution, on living organisms. This is particularly important in understanding the impact of these factors on human health. Model organisms can provide insights into the evolution of biological systems (Bosch, 2019). By comparing the genetics and biology of different model organisms, researchers can gain a better understanding of how organisms have evolved over time and how they have adapted to different environmental pressures. For instance, the study of the fruit fly has provided insights into the

evolution of the animal kingdom and the genetic basis of complex traits like behavior (Bosch, 2019). Some of the key discoveries in *C. elegans* are:

- Programmed cell death: *C. elegans* was the first organism where programmed cell death, also known as apoptosis, was discovered (Liu, 1999).
- RNA interference: The mechanism of RNA interference, or RNAi, was first described in *C. elegans*, leading to a Nobel Prize in Physiology or Medicine in 2006 (Zamore, 2006).
- Neuronal development: The development of *C. elegans* nervous system has been extensively studied, and the nematode has provided insights into neuronal differentiation, axon guidance, and synapse formation (Varier, 2011). Aging: *C. elegans* has been used to study the genetic and molecular basis of aging, leading to the discovery of many genes and pathways that regulate lifespan (Gao et al., 2018)
- Gene regulation: Many key regulatory genes and pathways were first discovered and characterized in *C. elegans*, including the insulin signaling pathway, the TGF-beta signaling pathway, and the Notch signaling pathway (Liu, 2004).
- Microbial interactions: *C. elegans* has been used to study interactions between host animals and microbial pathogens, and to identify genes involved in innate immunity (Alegado, 2003).

These are just a few examples of the many important scientific discoveries that have been made using *C. elegans* as a model organism.

The soil nematode *C.elegans*, when compared to mammals such as humans, has a unique immune system that is used to protect itself from pathogens (Apfeld, 2018). Their capacity to

survive, reproduce, and live in places where pathogens are abundant makes them very helpful for understanding pathogen-host interactions (Radeke, 2021). The goal of this literature-based and experimental project is to further understand what is currently known regarding how the nematode's immune system functions and find if there is a possibility that this knowledge can be applied or further explored in the human body.

Now, why should we care about NETs? NETs could be seen as members of the front lines of the immune system since NETs are formed once the pathogens enter an organism's system and neutrophils are recruited from the bloodstream as part of defense mechanisms (Rigby, 2012). Because of neutrophils' ability to immediately act upon attack, they attempt to capsule the foreign organism as quickly as possible to overcome the pathogen's attack. NETs might also have a deleterious effect on the host because the exposure of extracellular histone complexes could play a role during the development of autoimmune diseases (Brinkmann et al., 2004). These mutations or malfunctions, when they occur, can be seen as self-destructive or when they turn against their own host by attacking white blood cells.

An example of this would be their role in facilitating metastasis in cancer tumors or masses (Masucci, 2020). NETs play a key regulatory role in the tumor microenvironment, such as the development of distant metastases through the secretion of proteases (Demkow, 2021). This process not only facilitates metastasis but also increases the rate of proliferation of cancer cells throughout the body, enhancing the harm of the mutations caused by cancer cells (Demkow, 2021). On the other hand, these fibers could also work as the immune system's communication-like network assisting with communication or facilitating signaling transport. Unfortunately, in some cases, it could lead to a more harmful outcome (Matkó, 2021).

Autoimmune diseases such as lupus, or multiple sclerosis, take advantage of NETs' function of connecting a few elements within the immune system and use them as a shortcut to spread their genetic code more rapidly (Lee et al., 2017). These processes have caused lots of controversy among scientists regarding whether NETs are more harmful than helpful, a topic that has not been fully defined yet (Chapman et al., 2019). The neutrophil is a powerful defender, ejecting a 'net' of DNA, histones, and antimicrobial agents to kill pathogenic invaders, but failure to remove fragments of these NETs can lead to autoimmune diseases (Herre et al., 2023). The anatomical structure of this organism is shown in Figure 2. This figure is a visual that shows the location of leukocytes such as neutrophils in mechanisms like NETs. However, scientists have recently started to research this topic to reverse this effect and try to change NETs' function as an activator for promoting certain diseases, into an inhibitor that will help the body resolve the disease at a faster rate.

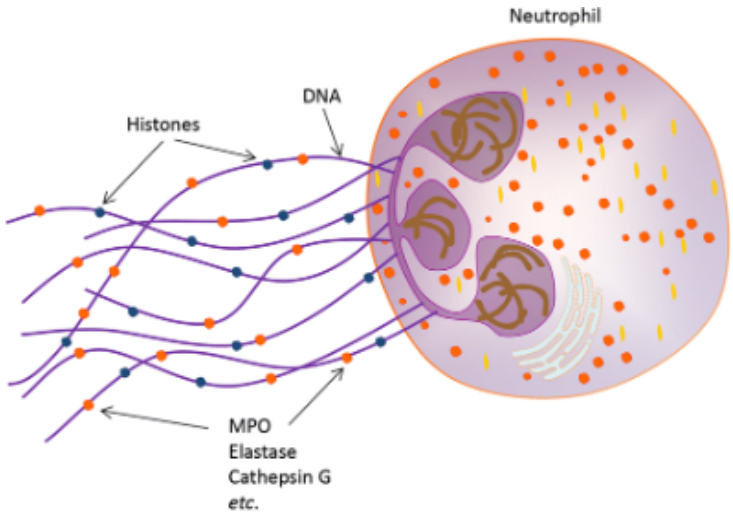


Figure 2. This image shows the structure of NETs. Figure from (Wang, 2021)

Another great discovery for NETs emerged during the Covid-19 pandemic. As scientists worldwide were exploring multiple solutions to the biggest problem on Earth, they discovered

that the NETs located in lung tissue were triggering an inflammatory response in an attempt to protect the lungs during a Covid-19 infection (Borges, 2020). After this realization, some immune systems' responses to different respiratory diseases were further analyzed to determine that, indeed, NETs played a major role in those inflammatory reactions (Law, 2017). The discovery of NETs' participation in the immune response against Covid-19 re-opened the door to a new world of exploration that has not been fully understood since the beginning of the study of the immune system in the 19th century. Increasing the amount of NETs expressed, using treatments like NET-targeting approaches, and promoting and enhancing their formation, could reduce the damage caused by hyperinflammation thereby decreasing the disease's severity and avoiding mechanical ventilation and consequently diminishing mortality (Borges, 2020). However, NETs would not directly target the new coronavirus. In simpler terms, NETs would not be the direct cure or form of treatment for a respiratory disease such as Covid-19, but theoretically, NETs would be able to trigger a series of chain reactions that would facilitate the way it is treated and increase the survival rate (Borges, 2020).

An effective way to measure NETs with the tools available currently is flow cytometry (Masuda et al., 2017). NETs can be measured by establishing a simpler objective and quantitative method for the detection of neutrophils that formed NETs using a plasma membrane-impermeable DNA-binding dye, SYTOX Green, in flow cytometry. Flow cytometry is a laboratory technique used to detect and measure the physical and chemical characteristics of a population of cells or particles (Masuda et al., 2017). Basically, in this process, a sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instrument. Commonly, flow cytometry studies are used to identify and quantify immune cells and characterize hematological malignancies. In the case of NETs, flow cytometry would be able

to show us the presence and the number of NETs present in vivo when exposed to certain scenarios such as peritonitis, which is the inflammation of the membrane lining the abdominal wall and covering the abdominal organs (Masuda et al., 2017).

NETs have been a controversial topic among the scientific community since originally they were defined as antimicrobial, which would mean they are helpful. The purpose of my thesis will be to find a response to this dilemma. I will be missing a lot of perspectives, due to limited time and resources, but the final results from the experimental portion will provide important insight into how we can (or cannot) use NETs' perplexing mechanism(s) to our advantage, opening a new topic of conversation that could potentially grow and become a great medical approach for patients with autoimmune diseases or health disorders that inhibit them from healing themselves in the case of a pathogenic infection.

Chapter 2: Neutrophil Extracellular Traps, *C. elegans*

The best, most accurate model to understand the immune response against pathogens in humans is the human body. Unfortunately, there are practical and ethical limits to investigating NETs' involvement in diseases in actual human bodies. This is where our little friend *Caenorhabditis elegans* comes into play. *C. elegans* is a free-living transparent nematode worm that lives primarily in soil. The transparency of their body allows observation and identification of the organs/organelles to be easier compared to other species (Keir, 2013), i.e. the fruit fly *Drosophila melanogaster*. This transparency would allow us to use the resources available at our university to look for NET formation. Later on, I will discuss the lab techniques that I used to obtain our desired results, along with a log/diary of the attempted approaches to find the best one. The results will take our limitations into account. The most impactful limitation we faced was the lack of time to perform this experiment, along with the lack of advanced equipment for analysis that might extend the amount of time spent in the lab as well as the non-perfect results obtained. In order to decrease the impact of those limitations, we would have to make sure to analyze our options and choose the one that presents the most accurate approach and seems to work with the tools and materials available at the Department of Biology at Regis University. In terms of visualization, fluorescence stains seemed to be the best analysis approach since they allowed us to observe desired structures for an extended period of time. Figure 3 is an exceptional representation of how *C. elegans* allows for the observation and identification of organs, organelles, and immunological pathways.

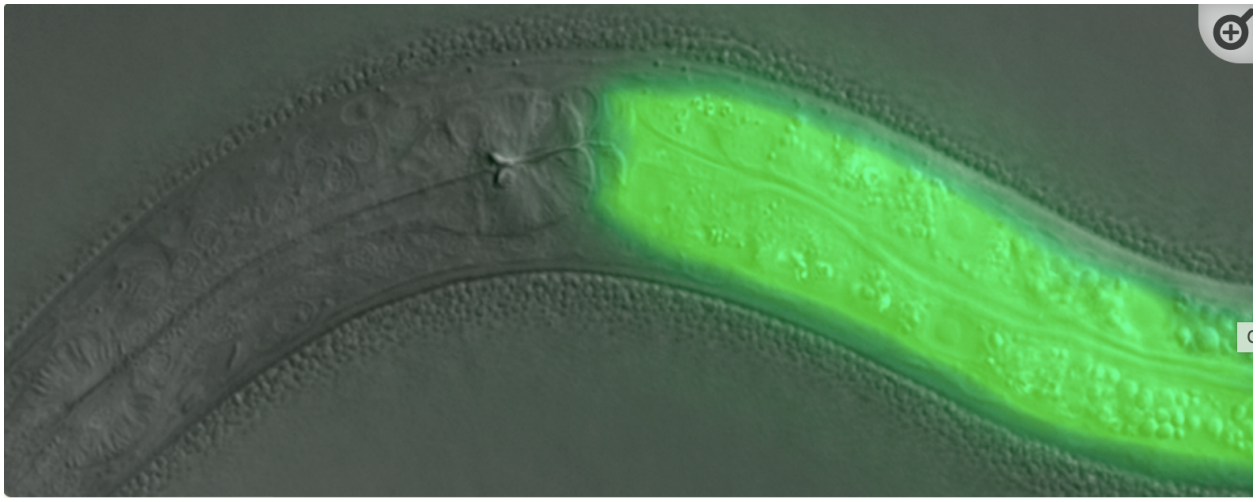


Figure 3. Depicted is a *C. elegans* hermaphrodite carrying a *lys-7::gfp* transgene. In this animal, GFP expression is controlled by the gut-specific lysozyme-7 promoter, meaning only the intestine is fluorescing. The image is an overlay of fluorescence and Nomarski images (images merged using Adobe Photoshop). Image adapted from Fig. 1. Copyright © American Society for Microbiology, Molecular, and Cellular Biology, 27, 2007, 5544–5553, doi:10.1128/MCB.02070–06.

Adding to these characteristics is that the *C. elegans* life cycle is relatively short, taking about three days for the animals to develop, with an overall lifespan of about two to three weeks (Apfeld, 2018). Facilitating its mass production and having an easier availability factor compared to other species makes them very attractive to researchers. Despite this small size, *C. elegans* has many of the organ systems present in more complex organisms, including a digestive system, nervous system, musculature, and reproductive system. This is the reason why *C.elegans* has been used as a model organism to study human diseases ranging from Parkinson's disease to mitochondrial diseases, as well as studying the immune system (Yokoyama, 2020). Adding to this, they can also eat, excrete, and mate (Apfeld, 2018). These are very instinctual functions of most living organisms, including humans.

Even though explaining the direct connection between *C. elegans* and humans is not the main purpose of this document, it is essential to understand how these two organisms are related.

To explain this, I had help from various experts in many biological fields, but the most important one was an evolutionary biologist. A phylogeny tree is a tool used to map the evolutionary relationship between organisms; therefore, we went ahead and created one. This phylogeny shows the relationships among multiple species, just to put in perspective *C. elegans'* anatomical and physiological structure compared to other species (Figure 4).

The phylogeny tree was created to show the evolutionary relationships between multiple species and to compare *C. elegans'* anatomical and physiological structure to those of other species. The traits used to build the phylogeny would depend on the specific organisms included in the analysis. Generally, phylogenies are constructed based on shared characteristics, such as physical features, genetic information, and behavior, that indicate common ancestry. As we can observe in the figure, *C. elegans* are quite similar to species like beadlet anemone or tiger worms. This phylogeny can serve as a map to identify how far away in similarity our model organism is from other species that may be known for having NETs formation

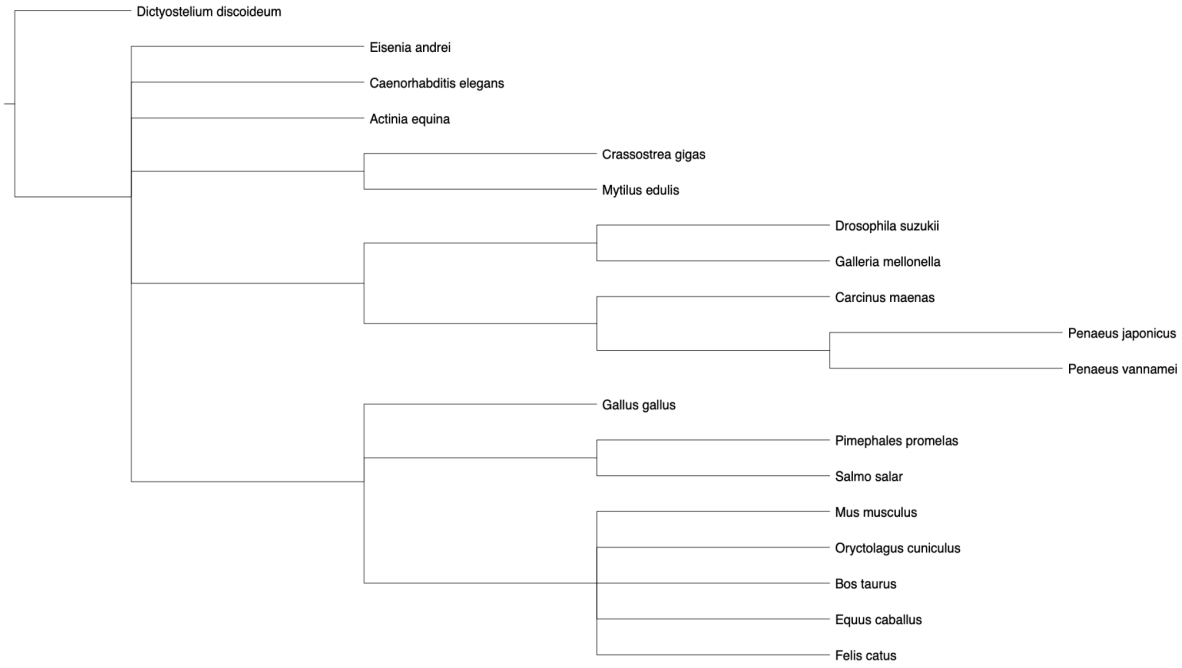


Figure 4. The phylogenetic tree of *C. elegans* compared with many other species is known to be helpful for research. By D. Carrasco, A. Spence, T. Imfield, B. Lucas, Regis University Department of Biology. Final draft. The website we used to generate that phylogenetic tree was the National Center for Biotechnology Information (NCBI) Common Tree tool: https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi.

In terms of sex, *C. elegans* exists as either of two sexes, a hermaphrodite or a male. This could be seen as a major difference in comparison to us, but it has a positive experimental side to it. The existence of self-fertile hermaphrodites has great advantages for the study of development because mutant stocks that would be unable to mate (such as paralyzed animals) are still able to self-fertilize (Apfeld, 2018).

C. elegans are very easy to maintain at research facilities. Due to their small size, nematode manipulations can be performed using a dissecting microscope, where individual nematodes can be transferred from plate to plate using a small platinum wire, which allows researchers to isolate individual hermaphrodites for self-fertilization. This can lead to large populations that allow investigators to set up crosses between the sexes. The small size of *C. elegans* means hundreds or thousands of animals can be maintained inexpensively on an individual dish. When the animals use up all the food, they will starve and can be maintained as starved populations for months. For long-term storage of stocks, nematodes can be frozen and kept in frozen vials for decades at −80°C or in liquid nitrogen (Keir, 2013). The small size, rapid life cycle, and amazing genetic and genomic tools available have made *C. elegans* a premier model organism for many purposes.

For all of the reasons mentioned previously, we have decided to use *C. elegans* as a model for our study, which consists of identifying and analyzing NETs in *C. elegans'* immune response. This will allow us to open new research doors and potentially use our protocols as the

first steps of new procedures to better understand the *C. elegans* immune response. *C. elegans* have six coelomocytes: large, ovoid, mesodermal cells situated as three pairs (right, left, and dorsal) in the pseudocoelomic cavity adjacent to the somatic musculature. Analyzing these cells is our best bet to obtain any results in the form of genetic material and encounter results. While we have some understanding of the functions of coelomocytes in C. elegans, there is still much that we do not know about these cells. One reason for this is that coelomocytes are a relatively understudied cell type in this organism compared to other cell types, such as neurons or muscle cells. Additionally, coelomocytes are difficult to study in C. elegans due to their small size, complex morphology, and the fact that they are located within the body cavity. Learning how to analyze these cells is at the core of this experiment.

Chapter 3: Immune Response, *C. elegans* vs Mammalian

The nematode *Caenorhabditis elegans* relies on its innate immune defenses to counter infection (Kim, 2018). The secretion of antimicrobial proteins represents an ancient mechanism of innate immunity that is found in both plants and animals (Ausubel, 2005). *C. elegans* have a very unique immune response. Infection with pathogenic microbes engages the host's innate immune system (Melo, 2012). Innate immunity comprises several distinct classes of proteins that mediate the multiple steps involved in a protective response to pathogen infection (Kawai, 2011). First, there are the host receptors that recognize the presence of a pathogen and/or pathogen-induced host damage (Janeway et al., 2001). Second, signal transduction pathways downstream of the initial recognition of infection require proteins such as kinases and phosphatases, as well as transcriptional regulators that direct changes in gene expression (Janeway et al., 2001). Third, antimicrobial peptides (AMPs) and proteins as well as secreted signals that coordinate an organismal response to infection serve as the effector mechanisms of the innate immune response to control infection (Kim, 2018). *C. elegans* have a more simplistic model in comparison to mammals, which allowed us to consider them as a model organism for this study.

Figure 5 below has a scenario of an infected specimen, including visuals to show how *C. elegans* are affected when exposed to different pathogens. The techniques presented in panel A are the most similar to what we performed in this experiment to identify the presence of NETs and determine if *C. elegans* undergo NETosis or not.

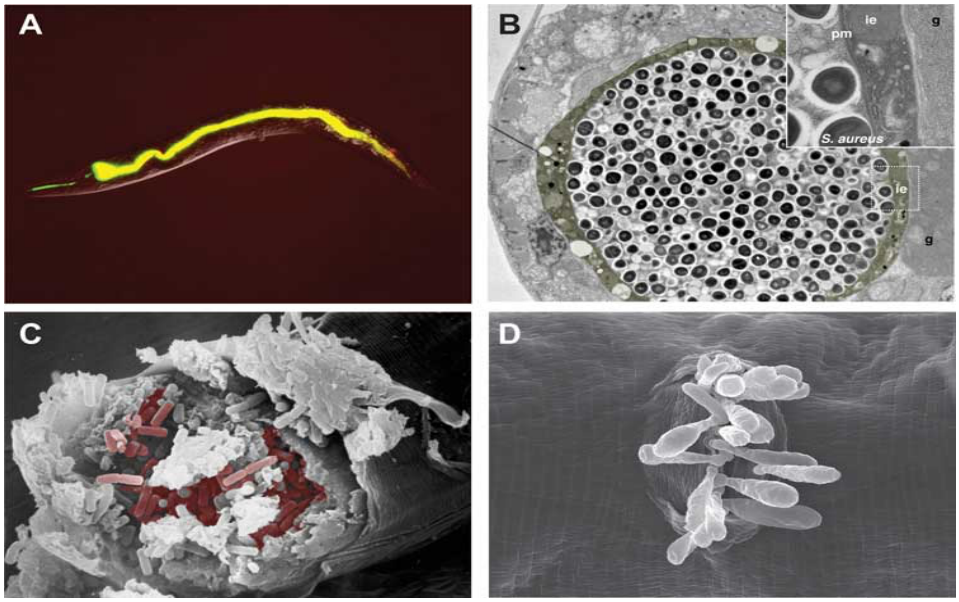


Figure 5. Models of infection in *C. elegans*. (A) Fluorescence micrograph of an adult hermaphrodite 30 minutes after having been placed on a lawn of *Enterococcus faecalis* V583::GFP. The intestinal lumen is distended and full of GFP-expressing bacteria. (B) Transmission electron micrograph of an adult hermaphrodite infected by feeding Staphylococcus aureus for 36 h. In this transversal section, the intestinal epithelium (ie, in yellow), and the gonad (g) are highlighted. The inset shows the boxed region at a higher magnification, illustrating the integrity of the plasma membrane (pm) and the dramatic loss of cytoplasmic volume in the intestinal epithelial cell. (C) Scanning electron micrograph of an adult hermaphrodite split open to reveal a large number of *Bacillus thuringiensis* vegetative cells colonizing the intestine. Some of the *B. 0thuringiensis* cells have been false-colored. (D) Scanning electron micrograph of an adult hermaphrodite showing multiple *Drechmeria coniospora* (strain JUf28) spores attached at the vulva. Original images courtesy of Hakkim Rahamathullah, Fred Ausubel, Javier Irazoqui, Hinrich Schulenburg, and Marie-Anne Félix. Figure obtained from Kim, D. H., & Ewbank, J. J. (2018). Signaling in the innate immune response. WormBook: the online review of *C. elegans* biology, 2018, 1–35.

On the other hand, the mammalian immune response is a bit more complex. In mammalian specimens, the innate and adaptive immune responses work in conjunction to attack pathogens in an attempt to clear infections (Chaplin, 2010). The mammalian immune response is more specialized than those organisms that belong to the phylum Nematoda such as *C. elegans* (Battisti et al., 2017). This immune response is capable of fighting pathogens through lots of

different pathways, and most importantly, it can produce memory immune cells that prevent or help for the quicker relief of future possible infections (Iwasaki, 2015). Among many cells produced by the immune system in mammals, the lymphocytes of the adaptive immune response are critical: T cells and B cells are the most important cells for survival (Cerutti, 2013). The B cells produce antibodies that attack invading bacteria, viruses, and toxins (Alberts et al., 2002). The T cells destroy the body's cells that have themselves been taken over by viruses or become cancerous (Overwijk et al., 1999). The T-cell receptor and B-cell antibody receptor, key components of the adaptive immune response, possess specificity that is generated randomly and selected clonally during T and B lymphocyte development (Thompson, 1995). Conversely, the pattern recognition receptors of the innate immune system have predetermined specificity, established through evolution, and play a crucial role in distinguishing self from non-self in the initial stages of an infectious response (Medzhitov, 1997). Below in Figure 6, we can appreciate a visual of what has been described. The figure shows a very simplified version of the immune system that shows us how the main components of the innate and adaptive immune response are leukocytes and T/B cells, respectively. This figure depicts the process by which the innate immune system employs specific leukocytes, in concert with macrophages and dendritic cells, to mount an attack on sites of infection. Subsequently, when antigens are presented to the host, the adaptive immune system is activated, leading to the generation of T and B cells, which are responsible for establishing immune memory.

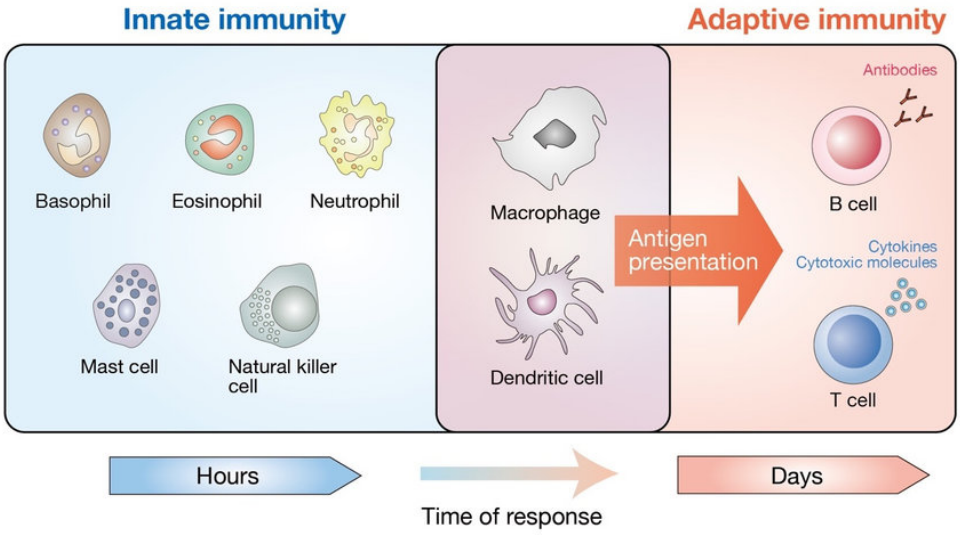


Figure 6. The main components of the innate and adaptive immune response respectively. The figure shows how the innate immune system uses specific leukocytes to attack sites of infection with the help of macrophages and dendritic cells. Later on, once antigens are presented to the host, the adaptive immune system is triggered resulting in the production of T and B cells. Cells that are in charge of creating memory immune cells. Figure obtained from Yamauchi, Takayoshi & Moroishi, Toshiro. (2019). Hippo Pathway in Mammalian Adaptive Immune System. Cells. 8. 398. 10.3390/cells805039.

Now knowing the differences between the mammalian and *C. elegans* immune responses, we can see how these two are related. They both share an innate immune response, meaning that *C. elegans* and mammals could share some components or pathways (Kurz, 2004). Thus, supporting our claim that *C. elegans* could be a very useful research model organism for our study and many more. Neutrophils are now considered complex cells capable of a significant array of specialized functions, and as an effector of the innate immune response they are able to regulate many processes such as acute injury and repair, cancer, autoimmunity, and chronic inflammatory processes (Liew, 2019). Nematodes lack circulating immune cells such as hemocytes of other invertebrates or neutrophils of vertebrates (Battisti, 2019). However, there could be a granulocyte or some sort of cell that has very similar characteristics to neutrophils. If

we were to find a similar granulocyte, it would be in the coelomocytes, since it is the only place where they could form based on the nematode's anatomical model. For example, chickens express heterophils to create HETs (Heterophil Extracellular Traps) as a defense mechanism against pathogens. Heterophils in avian species are the equivalent of neutrophils in humans (Boonlaos et al., 2021). Therefore, we want to be able to identify these structures in *C. elegans* since they have been shown to exist in other many living organisms of different species.

Coelomocytes are specialized cells found in the body cavity (coelom) of many invertebrates, including the nematode *C. elegans*. In *C. elegans*, coelomocytes are thought to play a role in a range of biological processes, including innate immunity, excretion, and development (Tahseen, 2009). This includes a diverse range of invertebrate species such as earthworms, sea urchins, snails, clams, crustaceans like shrimp and crabs, and tunicates (Buchman, 2014). We know that coelomocytes are highly phagocytic cells that can engulf and degrade foreign particles, including bacteria and other pathogens. They also play a role in the clearance of apoptotic cells and debris. Coelomocytes have been shown to express a range of genes involved in the innate immune response, including genes encoding antimicrobial peptides, lysozymes, and reactive oxygen species (Flannagan, 2012). Despite our understanding of some of the functions of coelomocytes, there is still much we don't know about them. For example, the molecular mechanisms by which coelomocytes recognize and respond to pathogens are not well understood. It is also unclear how coelomocytes interact with other cells in the *C. elegans* immune system, such as the epidermal and intestinal cells that also play a role in innate immunity. In addition to their role in immunity, coelomocytes are also thought to play a role in excretion, as they have been shown to take up and eliminate waste products from the body cavity. They may also play a role in regulating fluid balance in the body cavity, although this has

not been well studied (Cooper, 2002). Coelomocytes are a fascinating cell type in C. elegans with diverse functions that are still being uncovered. Their role in innate immunity, excretion, and development makes them an important model system for understanding the biology of invertebrates and the evolution of the immune response.

Chapter 4: Neutrophil Extracellular Traps, Other Species

The action of NETs formation is known as NETosis. NETosis is a program for the formation of neutrophil extracellular traps (NETs), which consist of modified chromatin decorated with bactericidal proteins from granules and cytoplasm. Various pathogens, antibodies and immune complexes, cytokines, microcrystals, and other physiological stimuli can cause NETosis (Vorobjeva, 2020). Before NETosis was defined, Etosis was the term used to explain this mechanism. This is because species use different types of cells unique to them, or sometimes shared with others, to create what is defined as extracellular traps.

ETosis was first discovered in mammalian neutrophils and is now known to occur in a variety of mammalian innate immune cells. The phenomenon entails the expulsion of chromatin from the nucleus via reactive oxygen species involvement. The discharged chromatin forms complex meshes that ensnare and kill bacteria, fungi, viruses, and other parasites (Robb et al., 2014). Thus, throughout the years, scientists were able to start naming these structures based on the cells present in the species found, inspiring scientists to look more into the topic with hopes of finding Etosis in other species.

The goal of the experimental portion of this thesis is to innovate and expand the knowledge we have acquired throughout the years as a research community. NETs in *C. elegans* have never been studied before. However, this immune response mechanism has been studied in other species. Some of the species where NETs have been found are not related to the physiological and anatomical composition of *C. elegans*. Some examples of these species are cats (felidae), chickens (galine), cows (bovine), horses (equine), and some plant species. To prove the existence or not of NETs in the nematode *C. elegans*, the spotlight focuses on those species with similar characteristics to those of the species we are concerned about. An

exceptional example of this is the parasite *Entamoeba histolytica*, commonly known as an amoeba. The presence of NETs in an organism like amoebas raises the possibility of support for our hypothesis. Figure 7 below provides some of the results obtained in the organism *Entamoeba histolytica* when analyzed to discover the presence of NETs. The figure shows the impact of *Entamoeba histolytica* on human white blood cells called neutrophils. The experiment was done by exposing the neutrophils to the parasite or a chemical and then observing the cells after 4 hours. The cells were stained to see changes in the DNA and the results were seen under different lights. *E. histolytica* shows that indeed it can induce NETosis. This raises the question: Do *C. elegans* have the ability to create NET formations?

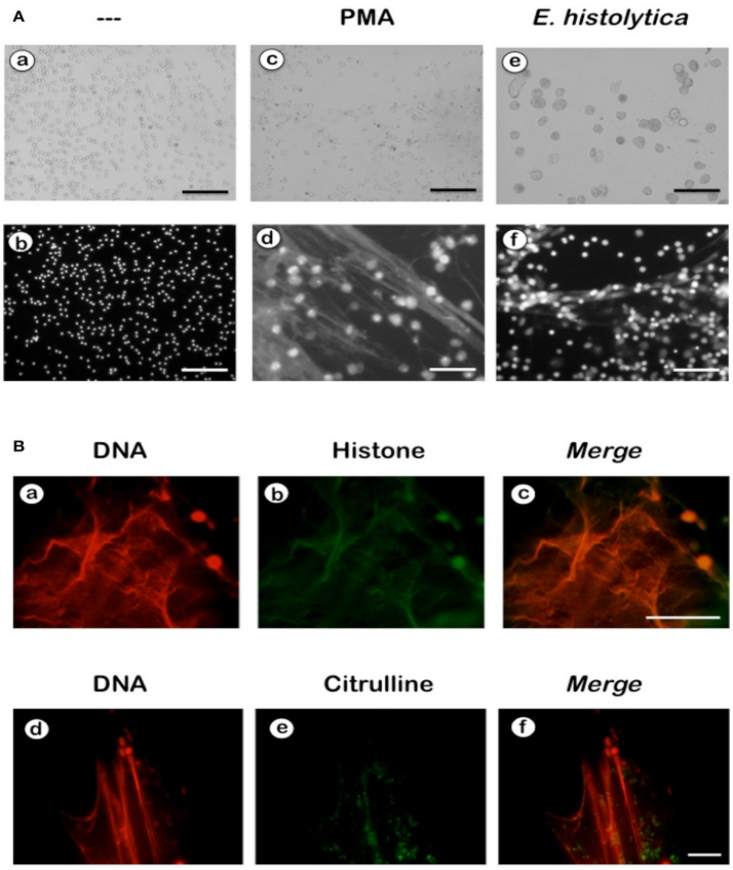


Figure 7. *Entamoeba histolytica* induces NETs formation. (A) Human neutrophils (PMN) were left untreated (—) or were stimulated with 20 nM phorbol 12-myristate

13-acetate (PMA), or by *E. histolytica* trophozoites (amoeba to PMN ratio 1:20). After 4 h, cells were fixed and stained for DNA with DAPI. Microphotographs were taken with white light (a,c,e) or with fluorescent light (b,d,f) and are representative of more than 10 experiments. The scale bar is 100 µm. (B) Human neutrophils were stimulated with trophozoites for 4 h at 37°C. Cells were fixed and immunofluorescence was performed using anti-histone H4 or anti-citrulline antibodies followed by TRITC-conjugated anti-rabbit IgG antibody. DNA was stained with DAPI. Scale bar 50 is µm. Figure from (Fonseca et al., 2018)
Note: Citrulline is an amino acid that is produced when certain proteins are modified through a process called citrullination. This process involves the conversion of the amino acid arginine to citrulline, which can alter the properties and functions of the proteins. In the context of the study, the detection of citrullinated proteins in the human neutrophils provides information about the effects of stimulation by Entamoeba histolytica trophozoites on the proteins in these cells

As we can observe from Figure 7, there are indeed very clear formations. The idea is to replicate these studies (as closely as possible) in order to identify whether there are NETs in *C. elegans*. One of the techniques that will be discussed in the next chapter was inspired by this study. Immunofluorescence consists of staining the cells in the organisms that might contain genetic material and have the potential of forming NETs. Also, the organisms should be fixed in order to be manipulated properly. This article was crucial for developing one of our techniques since they proved to be successful and managed to record the presence of NETs in an organism not that far away from our loved *C. elegans* (Zhang, 2016).

Looping back to the presence of NETs in different species, let's discuss the importance of NETs in these organisms. First, let's discuss how the study of other mammals has played a very important role in the development and survival of humanity. For example, chickens express an innate immune response against pathogens that show a similar mechanism to what we are expecting to find in *C. elegans*. Recent research in mammals and fish has revealed a fascinating mechanism by which neutrophil nuclear material associated with cytoplasmic granular content is released in the form of NETs, which can effectively trap and kill invading microorganisms both

in vitro and in vivo. In order to determine whether a similar defense mechanism is present in chicken heterophils, scientists utilized hydrogen peroxide (H2O2) and phorbol myristate acetate (PMA), a powerful activator of protein kinase C (PKC) enzymes which are involved in a range of vital cellular processes including cell proliferation, differentiation, and apoptosis. The study, conducted by Chuammitri in 2009, provides valuable insights into the potential existence of this mechanism in chickens, opening up new avenues for research in avian immunology (figure 8). Heterophils are the major phagocytic leukocytes in birds, with an analogous role to neutrophils in mammals**.** After a series of studies and analyses, heterophil extracellular traps were described for the first time in an avian species. The heterophil is the avian equivalent of the mammalian neutrophil. The release of extracellular fibers, consisting of DNA, histones, and granular enzymes, appears to be conserved in the innate immune system of phylogenetically diverse species (fish, birds, and mammals) (Chuammitri, 2009).

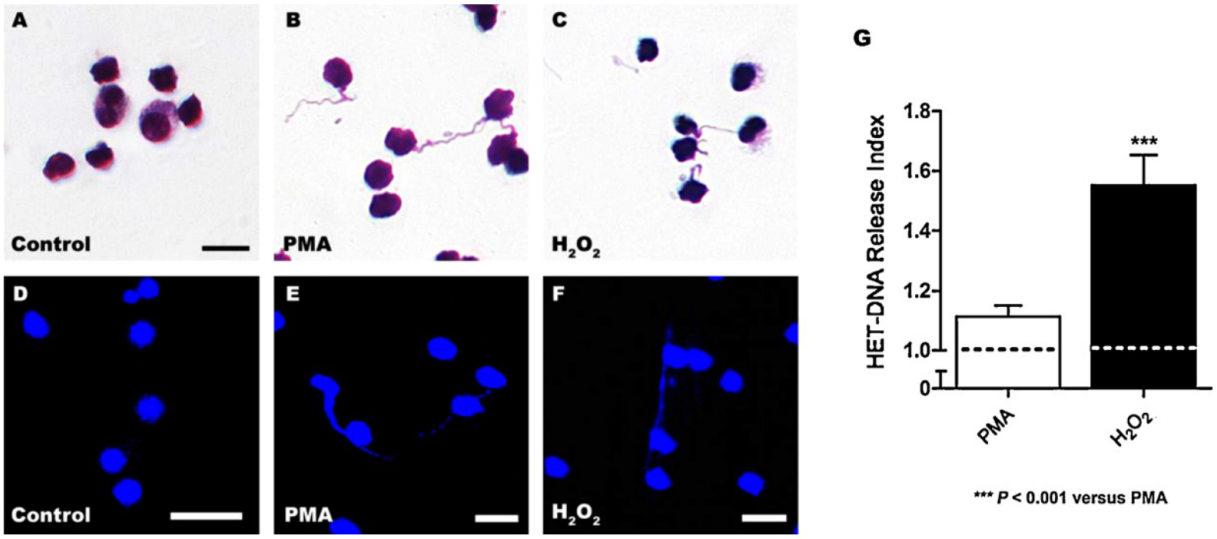


Figure 8. Chicken heterophils are cells that are found in chickens. When they are stimulated, they release something called HETs. Scientists used a special tool called confocal microscopy to look at the HETs and see how they were connected to the nuclei of the heterophil cells. They found that the HETs came from the center of the cells and were being released outside of the cells. They also tested how the release of

HETs changed when the cells were stimulated with PMA or H2O2. The results showed that more HETs were released when the cells were stimulated. The graph in (G) shows the increase in extracellular DNA release compared to non-stimulated cells, represented by dotted lines. Figure from (Chumaturri, 2009).

Similarly, ex vitro mucus samples of equine specimens that were prone to bacterial and viral infections were analyzed in an attempt to prove the presence of NETs (figure 9). To the best of our knowledge, this study shows for the first time that stimulated equine PMNs (A type of immune cell that has granules (small particles) with enzymes that are released during infections, allergic reactions, and asthma such as neutrophils) have the capacity to form NETs in vitro when in the presence of selected bacteria causing uterine infection in the mare, such as *E Coli*, Zoo, or Scap bacterias which are usually found in living organisms like horses due to their diet and natural habitat (Rebordão et al., 2014). Clearly, evidence of NETs' presence was obtained. Just like chickens, horses have the ability to produce these defense mechanisms upon activation/stimulation, which usually occurs when the specimen is undergoing a pathogenic threat. Furthermore, during this study, techniques such as DAPI staining also known as immunostaining were used. Thus, the methods mentioned could be also applicable to *C. elegans* analysis, if they are adapted correctly. One of the objectives of my study is to find a technique that will allow the identification of NETs in *C. elegans* and serve as baseline experimentation for future research.

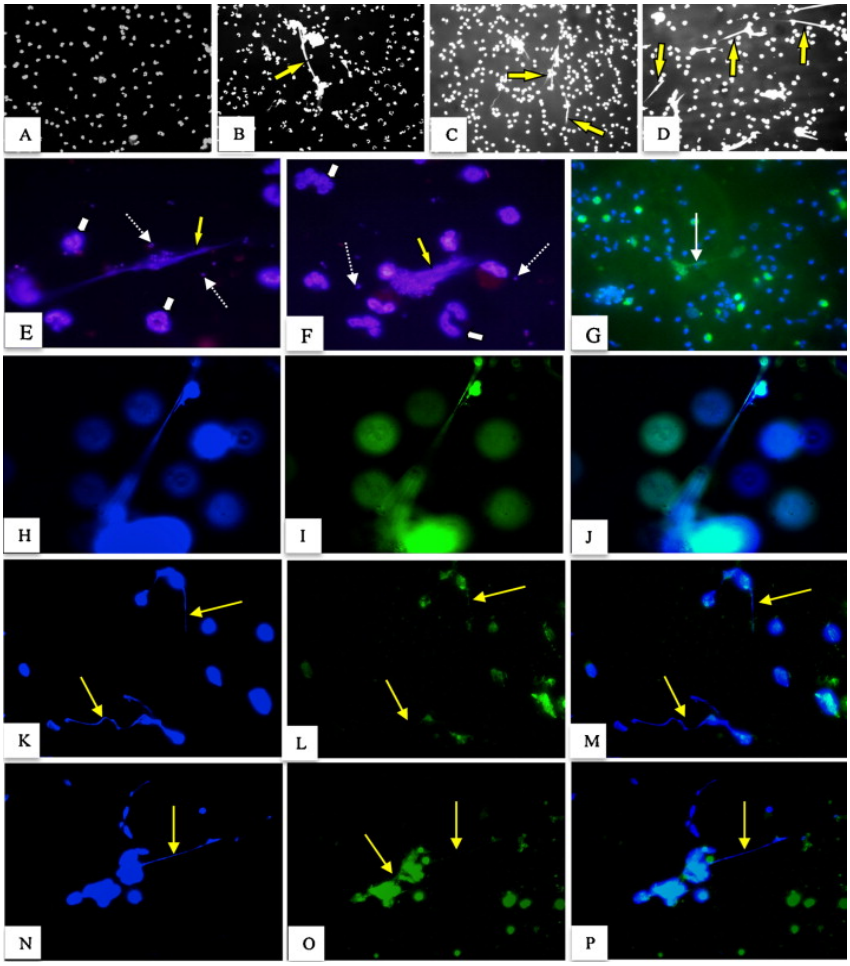
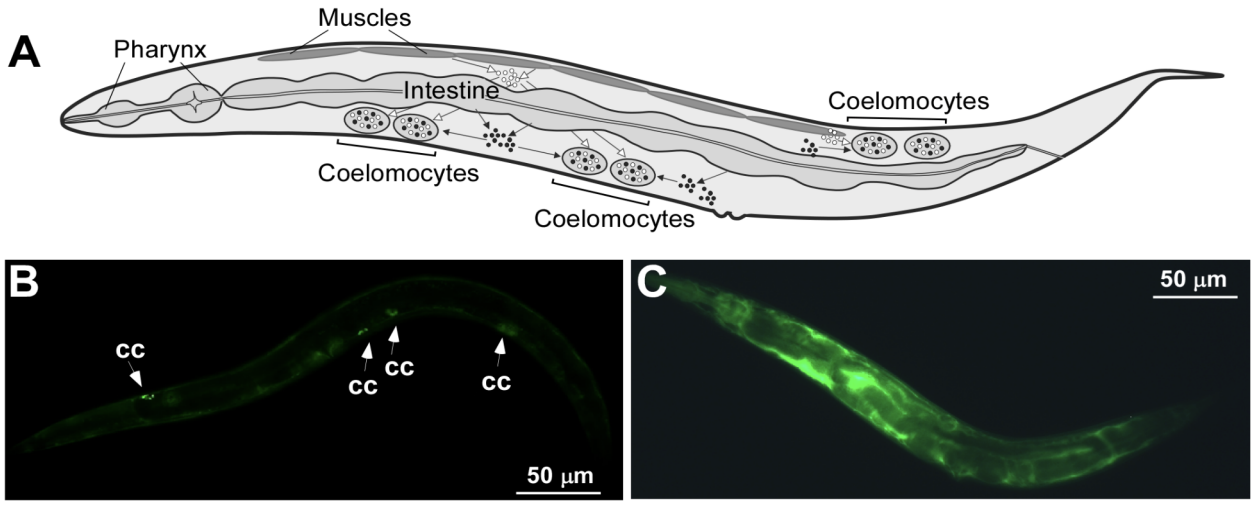


Figure 9. In vitro (A–G) and ex vivo (H–P) neutrophil extracellular traps (NETs; yellow arrows) released by equine neutrophils (PMN). NETs induced after in vitro PMN stimulation with phorbol-myristate-acetate (PMA) and mare endometritis bacteria: A: PMN alone (negative control); B–D: after 1-h (B), 2-h (C) or 3-h (D) incubation with 25 nM PMA (Mag = 200×); E: PMN (thick arrow) stimulated with 25 nM PMA and Staphylococcus capitis (dashed arrow); F: PMN stimulated with 25 nM PMA and cytochalasin, a phagocytosis inhibitor and Staphylococcus capitis (dashed arrow; Mag = 1000×). A–F: NETs were stained with 4′,6-diamidino-2-phenylindole (DAPI). G: Histone H2B positive immunostaining of equine in vitro NETs (white arrow) confirms the nuclear origin of the extruded material (Mag = 400×). H–P: Ex vivo NETs obtained from equine endometrium with endometritis stained with DAPI (H, K, N) and immunostaining for histone H2B (I; Mag = 400×), myeloperoxidase (L; Mag = 200×), and neutrophil elastase (O; Mag = 200×); right panels (J, M, P): merged. (Rebordão et. al, 2014)

As we have seen thus far, the identification of NETs has been studied in many well-known species. The next step for researchers is to explore the immune responses of those species that have very different, but useful responses against pathogens. The phylum Nematoda for example has very little research done on this topic. Based on the benefits found in this phylum, previously mentioned, scientific results could be obtained more quickly and safely than research on mammals. The protocols, research, and application of immunological terms presented in the species mentioned before were an inspiration to reach the final goal of this research study. Whether the study is successful or not, we can at least expect to open the door for further research, and an accidental identification of other immunological pathways may be found in the process.

Chapter 5: Neutrophil Extracellular Traps, Experimental Protocols

To further investigate the presence of NETs in *C. elegans*, different protocols had to be created and attempted in the lab. There is no record of previous studies in this species, so the lab protocols had to be created from scratch. Therefore, members of the Department of Biology at Regis University created the following protocols created based on successful analyses of NETs in other species or experimental procedures, with the exception that these were adapted to our specific goal. As previously mentioned in Chapter 2, *C. elegans* possesses six coelomocytes. The coelomocytes are large, ovoid, mesodermal cells situated as three pairs (right, left, and dorsal) in the pseudocoelomic cavity adjacent to the somatic musculature (Figure 10).



doi: https://doi.org/10.1371/journal.pone.0009564.g005

Figure 10. A schematic drawing of a worm shows that fluids secreted into the pseudo coelom from surrounding tissues accumulate in coelomocytes (A, modified from Fares and Greenwald, 2001 [18]). Epifluorescence micrographs of GS1912 (B) and NP717 (C) worms. In GS1912, GFP is expressed in body wall muscles from *myo-3* promoter, secreted into the pseudocoelom, and accumulated in coelomocytes [18]. White arrows indicate the accumulation of GFP in coelomocytes (cc, B). As a result of coelomocyte ablation in NP717, GFP accumulates in the pseudocoelom [18] (C). Figure obtained from (Schwartz et al., 2010).

The analysis of these cells may be the key to obtaining any results. We want to prove if coelomocytes are involved in immune functions. The model nematode *C. elegans* lacks an adaptive immune system and the coelomocytes are capable of endocytosis, but their involvement in phagocytosis of bacteria seems unlikely (Qudsia, 2009). The main goal of the protocols was to analyze the coelomocytes further and describe their potential role in the immune response, with the hopes of identifying NETs' presence.

After discussing many approaches, we decided to perform freeze-cracking, tube staining, and also live imaging using beads/levamisole. Tube staining would allow us to identify the coelomocytes and observe their interactions when infected with a pathogen. The freeze-cracking technique is a bit more complex than that. It consists of introducing a specific antibody to the nematode, which would later be observed via the fluorescence of the antibody. Once any or both techniques demonstrate to be successful or not, we can start defining the mechanisms involved in these interactions. Our hypothesis states that the formation of NETs might be a possible defense mechanism present in the nematode *C. elegans* immune system.

The Freeze-cracking technique:

Freeze-Cracking

In order to have enough for 1-2 slides, allow 1-2 medium plates of worms (*C. elegans)* to grow until the plates are covered with embryos

Preparation:

- Get dry ice (the amount recommended, around 2 lbs) and cover with an aluminum block.
- Place two glass slide holders in the -20C freezer by the door (of the freezer) and fill one with 100% methanol and the other with 100% acetone. Make sure to keep them separate.
- Create solutions that will be used:

    - At least 500mL PBST
        - 500uL Tween-20
        - 50mL 10X PBS
        - 450 mL ddH2O
        - **rotate to disperse the Tween-20 properly
    - PBST + 0.75% dry milk
        - 0.0750g dry milk
        - 10mL PBST
        - **Place the Falcon tube on the Nutator to dissolve the dry milk
    - Bleach solution (at least 8mL for each sample of worms will be needed)
        - 1mL bleach (Clorox could be used)
        - 1mL 10N KOH
        - 6 mL ddH2O
    - Make poly-L-Lysine slides:
        - Add 25uL poly-L-lysine to each ring on a ring slide
            - Let dry for at least 10 minutes
            - Use a Kim wipe to remove the remaining liquid
        - Use a razor blade to etch a description of the sample onto each slide

Antibody staining day 1:

- Bleach worms to obtain the embryos
    - Wash worms and embryos off the plate with M9 into a 15mL tube
        - A Pasteur pipet could be used to scrape the embryos off the plate or piper vigorously with a P1000 to get as many embryos as possible

    - Spin down the worms (~30 seconds on setting 3) and remove the supernatant.
    - Add 4mL bleach solution to the 15mL tube, spin down, and remove the supernatant.
    - Add 4mL bleach solution to the 15mL tube, and let the tube to nutate for ~5 minutes, or until the worms are dissolved and the embryos are free in solution.
    - Spin down the embryos and remove the supernatant.
    - Wash the embryos at least, and remove all but ~100uL of ddH2O from the embryos
- Add 50uL embryos in ddH2O to each ring of a poly-L-Lysine slide
    - Let the embryos settle for ~5 minutes
- Cover the rings with a 22 mm coverslip and use a Kim wipe to remove as much liquid as possible from underneath the coverslip.
- Freeze the slide on the aluminum block on dry ice for at least 20 minutes (It can be done in 15 minutes, especially when there are multiple slides to do)
- Remove the coverslip by using a razor blade to pry up one corner and then pop the rest of the coverslip off. **Next step must be done immediately.
- Transfer the slides to 100% methanol at -20C for 5 minutes. **Do not let cracked slides sit on dry ice.
- Transfer slides to 100% acetone at -20C for 5 minutes. **You need to be very precise about the time here as well.
- Wash slides for 3~5 minutes in PBST while rotating.
    - Fill a petri dish with PBST
    - Immerse the slide in the petri dish

    - Put Petri dishes on the rotator and turn it on to setting 2
- Prepare the primary antibody mix by diluting the antibodies to their appropriate concentration in PBST + dry milk.
    - Dry the slides off everywhere except in the rings
    - Add 20uL primary antibody mix to each ring
    - Place a 22mm coverslip on top of each ring
- Store slides overnight at 4C in a humidifier chamber
    - Add PBST to the bottom of a large Petri dish
    - Place a medium size Petri dish lid in the large Petri dish
    - Place the ring slide on top of the medium lid
    - Cover with the large Petri dish lid

Antibody Staining Day 2:

- Retrieve slides from 4C
- Analyze slides
- Identify the presence of fluorescent bodies due to antibody staining
- Obtain pictures and label antibodies through fluorescence microscopy

Tube Staining technique:

Tube staining protocol (Bethany Lucas)

Reagents needed:

- 4% Paraformaldehyde fix (make just before starting)
- 4.8µL 1M PIPES, pH 6.8 (final concentration 48mM PIPES)
- 200µL 50mM EGTA, pH 8.0 (final concentration 10mM EGTA)
- 5µL 0.5M HEPES, pH 6.8 (final concentration 25mM HEPES)

- 2µL 1M MgCl2 (final concentration 2mM MgCl2)
- 784µL ddH2O
- 2.5µL 5M NaOH (final concentration 13mM NaOH)
- 0.04g paraformaldehyde (final concentration 4% PFA)
- (Here, incubate for 5-10 minutes at 65°C, vortexing every few minutes until clear)
- Add 2µL Triton-X-100 (final concentration 0.2% Triton-X-100)
- Preparing poly-l-lysine slides (day 2)
- To ringed antibody slides, add 30µL poly-l-lysine and spread with the tip until filling the entire ring
- Incubate at room temperature next to flame for 30 minutes until slides turn white
- Rinse gently with 1mL PBS

Day 1 (~1:30-2:00 hrs)

1. Wash worms off plates gently with ddH2O into a 15mL conical tube; rinse 2X with ddH2O
2. Remove most of the liquid and transfer worms in approximately 150µL ddH20 to the Eppendorf tube
3. Add 200µL fix; incubate at room temperature for 20 minutes
4. Spin down for 2 seconds using a centrifuge; remove supernatant
5. Wash 3x 5 minutes with PBST; all washes are 1mL in volume throughout the protocol. Remember to spin down before removing the supernatant every time J
6. Block for 30 minutes in PBST + 0.75% non-fat dry milk
7. Remove supernatant so there's 50-100µL blocking solution in the Eppendorf tube (compare to reference tube); add diluted primary antibody to tube

8. Incubate overnight at 4°C, nutating or rotating

Day 2 (~3-4hrs)

1. Wash 3x 5 minutes with PBST
2. After the final wash, remove the supernatant down to 50µL (compare to the reference tube)
3. Add 1µL secondary antibody and/or 2µL phalloidin-488 (or 4µL phalloidin-555)
4. Incubate covered at room temperature for 2 hours, nutating; prepare poly-l-lysine slides during this incubation
5. Wash 2x 5 minutes PBST
6. Wash 2x 5 minutes PBS
7. Remove supernatant to the 50µL mark and pipet up and down to break apart worm clumps. Pipet the worms onto the poly-l-lysine slides.
8. Let worms settle for approx. 5 minutes
9. Remove excess liquid using a Kim wipe
10. Add 11µL slow-fade; seal with clear nail polish
11. Analyze slides
12. Identify the presence of fluorescent bodies due to antibody staining
13. Obtain pictures and label antibodies through fluorescence microscopy

Before going in-depth with the results of this experiment, I will share my experience to provide guidance for future researchers on this topic. Let me elaborate on the journey of the development of this research experiment that eventually became my thesis. Three and a half years ago Dr. Sterling approached me with an intriguing idea: would a nematode - in our case *C. elegans* - have innate immunity defense mechanisms such as NETs? This question had a big impact on my inner researcher. I began to feel genuine curiosity for the first time, I felt like I needed to do something about it and to find an answer. However, the execution of the research was interrupted by several events including the COVID-19 pandemic, and thus, it was not possible to effectively complete it at that time. Fast forward a few years, and I was finally able to take on this project as my honors thesis. I knew from the outset that this would be a challenging feat, especially considering the limited time frame. Nevertheless, I was determined to see it through.

With the help of Dr. Sterling's expertise in immunology and Dr. Lucas' expertise in *C. elegans*, we were able to collaborate effectively and bring the study to fruition. Throughout the process of working on this thesis, I gained valuable insights and knowledge in the field of immunology and came to appreciate the complexity and significance of the research. Furthermore, I was able to develop a deeper understanding and connection with creatures such as *C. elegans* through my research. In order to test our hypothesis, my advisor Dr. Lucas and reader Dr. Sterling and I met on a weekly basis to brainstorm various approaches. Prior to the experimental portion of the study, we received crucial support from Dr. Sterling and Dr. Imfeld in putting together a phylogeny tree to represent the relationship between humans and *C. elegans*. This allowed us to use the existing literature and find the most appropriate protocols for our study. After careful consideration and research, we determined that tube staining and

freeze-cracking techniques would be the best methods for executing our study. This required us to order specific worms and laboratory materials since they were not part of the stock materials in any of our research labs. After ordering specific kinds of *C. elegans*, specific beads to hold the *C. elegans* in place for analysis, and DAPI, we were able to start the experimental portion.

The hardest part at the beginning was the transfer of worms since it has to be done with a microscope and a very steady hand to prevent them from dying when being transferred. After practicing for a couple of weeks, I was able to master the techniques required for this and I was ready to start practicing with the protocols. Dr. Lucas was of great help since she stayed with me on multiple occasions to make sure I was doing everything correctly and understanding the meaning behind each step of the protocols. Luckily, I was able to perform both techniques successfully in terms of hands-on work. Unfortunately, the biggest challenge was to analyze the plate with the stained worms due to the lack of efficiency of the fluorescence microscope in the Biology department of my university. We suffered a cyberattack in 2019, and many lab instruments were affected by this. It has been really hard to restore them to their original efficacy. Ocular is the name of the software that we were planning on using. This would allow us to take very detailed pictures of the stained worms and to do it for extended periods of time aiming to look for specific formations the fluorescent coelomocytes would show. It was definitely a big challenge since we could not record the successful results obtained from both techniques. Luckily, the Neuroscience department has an identical microscope with more updated software that worked perfectly fine and allowed us to record data for analysis.

To wrap up my journey, it is important to emphasize that lack of time was the biggest limitation we faced to make this experiment a more extensive and precise study. I began the literature review for this experiment about a year and a half ago. Having a solid literature-based

research background was crucial to understanding this topic and it is definitely demonstrated in this paper. When the time to proceed to the experimental portion of it arrived, it was almost December, right before my last Christmas break as a college student, leaving only a couple of months to finish both, the experiment and a well-written document. I realized that it would not be enough time to obtain enough results. However, I did cover a big chunk of the study. This document could serve as a guide for future steps that I hope I can take over one day in the near future. Opening the doors for the research community to answer this question was my greatest goal when I started this project. Hopefully, I was able to provide the tools necessary for anyone who wants to take over this project and have a more meticulous approach that would show the validity of our claim.

Chapter 6: Results and Discussion

Neutrophil extracellular traps (NETs) have been found in mammals and fish and are known to trap and kill invading microorganisms by releasing nuclear material associated with cytoplasmic granular content. With recent research suggesting that these NETs may also play a role in the immune response of *C. elegans*, I sought to investigate the existence of NETs in the nematode. Using fluorescent dyes to stain DNA and bacterial cells in *C. elegans*, I imaged the nematodes using confocal microscopy to visualize the interaction between the DNA and bacteria. My hypothesis was that if NETs were present in *C. elegans*, then I would observe the release of nuclear material to trap and kill the bacteria. Unfortunately, I was not able to obtain any conclusive results from the experiment. However, we did prove part of our hypothesis by successfully tagging the coelomocytes. The next steps would be to infect these cells and take a closer look to seek for any kind of NETs formation (if any) and record the data. The limitations of the instruments and the short amount of time I had for the study may have contributed to the lack of results. Despite this setback, I was able to gain valuable insights into the biology of *C. elegans*. I learned based on the literature research, that *C. elegans* appear to have a unique immune system that is distinct from that of mammals and other model organisms. Specifically, I found that *C. elegans* lacks certain immune cells and signaling pathways that are present in other organisms. *C. elegans* possesses a primitive immune response that involves physical barriers, such as the cuticle and the intestinal epithelium and the secretion of antimicrobial peptides by the epithelial cells. However, *C. elegans* lacks the adaptive immune response found in mammals, which involves the production of specific antibodies and activating T and B cells. In terms of signaling pathways, *C. elegans* has a simple nervous system that allows it to respond to

environmental cues and coordinate its behavior. However, it lacks many of the signaling pathways found in higher organisms, such as the insulin/IGF-1 pathway and the MAPK pathway, which play key roles in regulating growth, metabolism, and stress response in mammals. This suggests that the nematode may have evolved its own unique mechanisms for combating invading microorganisms. Overall, while I was not able to prove the existence of NETs in *C. elegans*, my study highlights the importance of model organisms in scientific research. Even when experiments do not produce the desired results, they can still provide valuable insights into the biology of the organism being studied. Furthermore, limitations in equipment and time can be important factors in the outcome of an experiment, and as researchers, we should always take these factors into consideration when designing experiments**.**

Chapter 7: Conclusion

Neutrophil extracellular traps (NETs) are structures composed of DNA and associated proteins, which are released by neutrophils to trap and kill pathogens (Ramos-Kichik, 2009). NETs have been identified in a range of organisms, including mammals, but their presence in the nematode *C. elegans* had yet to be confirmed. The aim of this study was to determine whether NETs exist in *C. elegans* and to investigate their potential role in the nematode's innate immune response. Unfortunately, due to a shortage of time, we were unable to definitively prove the existence of NETs in *C. elegans*. Despite multiple attempts to visualize and isolate NETs using various methods, we were unable to obtain consistent results. The absence of definitive proof for the presence of NETs in *C. elegans* raises questions about the evolution and role of these structures in invertebrates. The literature reviewed in this thesis suggests that NETs are a relatively recent development in evolution, appearing only in jawed vertebrates (Heeb, 2020). It is possible that NETs have not evolved in nematodes due to their simple anatomy and efficient innate immune responses. Despite our inability to confirm the presence of NETs in *C. elegans*, this study sets the stage for future research on the evolution and role of these structures in invertebrates. Further research using alternative methods may be necessary to determine whether NETs exist in *C. elegans* and to understand their potential role in the nematode's innate immune response. Methods such as quantifying the release of extracellular DNA using a colorimetric or fluorescent assay. For example, the Quant-iT PicoGreen assay can be used to measure the amount of extracellular DNA in *C. elegans* culture media, which can serve as a surrogate measure for NETs release (Chortis et al., 2018). Additionally, genetic and molecular approaches can be used to study the regulation of NETs formation in *C. elegans*. For example, RNAi-mediated knockdown of genes involved in the innate immune response can be used to

investigate their role in NETs formation, while transgenic strains expressing fluorescently labeled NET components can be used to track NETs formation in real-time (Kawli, 2008). In conclusion, the lack of time in this study was a significant obstacle in definitively proving the existence of NETs in *C. elegans*. Nevertheless, the literature reviewed in this thesis provides valuable insight into the evolution and potential role of NETs in invertebrates and serves as a foundation for future research in this field.

However, we have provided enough evidence to understand why *C. elegans* are a great model for scientific research. *C. elegans* is a nematode worm that has become a popular model organism in biomedical research (Kaletta, 2006). Over the past several decades, scientists have discovered that *C. elegans* has several characteristics that make it an excellent tool for studying various aspects of human biology, including genetics, anatomy, physiology, and development. One of the main reasons that *C. elegans* is a great model for medical research is its well-defined and simple genetics. *C. elegans* has a small genome size and a short lifespan, which makes it easier for scientists to study the effects of genetic mutations and manipulations (Leung, 2008). By changing specific genes in the worm, researchers can observe the effects on its development, physiology, and behavior, which can then be compared to similar changes in humans. This information can help to shed light on the underlying mechanisms of various diseases and disorders, as well as the effects of different therapeutic interventions. Another advantage of using *C. elegans* as a model organism is its simple anatomy. *C. elegans* is a transparent worm with a simple body plan that consists of 6 big round cells called coelomocytes, making it possible to observe its anatomy and internal organs in detail. This simplicity also makes it easier to study the effects of drugs or other therapeutic interventions on its development and physiology. In addition to its simple genetics and anatomy, *C. elegans* has a well-characterized physiology, including its

innate immune system (Kim, 2018). Scientists have discovered that *C. elegans* has a highly efficient innate immune system that is capable of recognizing and eliminating pathogenic bacteria. By studying the molecular mechanisms of this immune response, researchers can gain insights into the underlying mechanisms of human immunity and the development of new treatments for infectious diseases. Finally, *C. elegans* is a versatile model organism that can be used to study a wide range of biological processes, from the molecular mechanisms of development to the effects of aging and neurodegeneration. Its small size and short lifespan make it an ideal model for high-throughput experiments, and its simple genetics and anatomy make it possible to perform experiments with a high degree of precision and control. In conclusion, *C. elegans* is a great model for medical research due to its simple genetics, anatomy, physiology, and development, as well as its versatility and ease with which it can be used for high-throughput experiments. By using *C. elegans* as a model organism, researchers can gain valuable insights into a wide range of biological processes and the underlying mechanisms of various diseases and disorders, which can help to advance the development of new treatments and therapies.

In conclusion, while my experiment did not yield conclusive results, the potential of NETs to combat infectious diseases like COVID-19 cannot be ignored (Schönrich, 2020). The release of nuclear material to trap and kill invading microorganisms can be a powerful tool in the fight against viral infections. Additionally, recent studies have shown that NETs play a crucial role in sepsis, a life-threatening condition that can occur in response to bacterial infections (Shen, 2017). By understanding the mechanisms behind NET formation and their role in immune response, we can potentially develop new therapies and treatments for these and other diseases. Furthermore, the study of NETs in model organisms like *C. elegans* can provide valuable insights into the immune systems of humans and other mammals. As I observed in my own

study, the unique immune system of *C. elegans* suggests that the nematode may have evolved its own mechanisms for combating invading microorganisms. By studying the similarities and differences between the immune systems of different organisms, we can better understand the complexities of the immune response and potentially develop new strategies for treating and preventing diseases. In light of the ongoing COVID-19 pandemic, the study of NETs and their potential applications in disease treatment is more important than ever. By continuing to explore the role of NETs in the immune response and their potential as therapeutic tools, we may be able to develop new treatments and therapies to combat infectious diseases and save lives.

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