5-2023

Characterization of Neutrophil Extracellular Traps in Naked Mole-Rats: A Step Towards Cancer Resistance

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CHARACTERIZATION OF NEUTROPHIL EXTRACELLULAR TRAPS IN NAKED MOLE-RATS: A STEP TOWARDS CANCER RESISTANCE

by

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Capstone submitted in partial fulfillment of the requirements for graduation with

University Honors

with a major in
Human Biology
in the Department of Biology

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Spring 2023

Abstract

Cancer is the second leading cause of death in the United States, exceeded only by heart disease. One of every five deaths in the United States is due to cancer. A growing area of research involves the analysis of cancer resistant traits in other species to understand their biological mechanisms and eventually make translations to human cancer research and clinical treatment. Because of their remarkable cancer resistance, the *naked mole-rat* (NMR) is a prime subject for this research, and various studies have already suggested that the immune mechanisms of the NMR might be harnessed for human cancer therapies[1-4,7].

In both humans and NMRs, neutrophils are the most abundant type of immune cell. As part of the human immune response neutrophils release *neutrophil extracellular traps* (NETs) in a process referred to as NETosis[8]. The ejected NETs are a matrix of DNA and histone proteins that function as extracellular webs to trap and destroy pathogens[9]. Unfortunately, NETs also worsen the effects of many diseases, and increasing evidence significantly links NETs with cancer[5,9,13-20]. When cancer cells are surrounded by NETs, they are afforded a variety of benefits. NETs help improve tumor vascularity by creating space for blood vessels while simultaneously forming a physical barrier that shields tumors from the cytotoxicity of other immune responses. This protection and blood supply markedly improve tumor growth, local invasion, and metastasis to other locations[13,15,16,19]. These results are from studies involving both mouse models and human tissue samples, but the literature is void of any publications regarding the character of NET release in NMRs.

The object of this study was to establish a protocol for isolation and NETosis induction of NMR neutrophils and characterize NMR NET release in comparison to humans for the first time. We hypothesized that NMR neutrophils would be less sensitive to NET formation compared to human neutrophils and that a higher concentration of stimulants will be required to induce NETosis in NMR cells. Working with scientists in the Schiffman Lab at the University of Utah Huntsman Cancer Research Center, we first established a protocol for the isolation, stimulation, and quantification of human neutrophil NETosis. With this protocol, NMR blood was collected and then separated via cell sedimentation and density gradients to isolate neutrophils. Cells were counted and seeded onto plates for the stimulation of NETosis with the bacterial toxins ionomycin and lipopolysaccharide (LPS). Results were collected via a Sytox Green fluorescence assay and confocal microscopy. We used neutrophils isolated from human blood as an experimental control. We were able to successfully isolate NMR neutrophils, stimulate and characterize NETosis, and compared it to human neutrophil control. Strikingly, our preliminary data does indicate increased resistance to NET release in NMRs compared to humans. Although more sampling will be necessary to further confirm and quantify these differences, this initial success nonetheless helps to confirm the value of researching NMR NETosis and shows promise for future experiments.

For my wife Jessi.

Acknowledgements

This project would not have been possible without the immense support and invaluable mentorship of Dr. Zhongde Wang. His guidance, suggestions, and enthusiasm throughout my time in his lab has made a profound impact on not only my undergraduate experience but will be felt throughout my career. The opportunities that he has given me have helped me to grow my love of research and deepen my understanding of the scientific process.

I would also like to take the opportunity to thank Dr. Yanan Liu, a postdoctoral fellow who has been there every step of the way throughout my time in the lab, and especially with this project. Yanan has taught me countless lab skills with patience, helped to conduct numerous experiments, given invaluable feedback, and overall made significant contributions to my work.

Additionally, to our collaborator from the University of Utah I express deep gratitude. Dr. Lisa Abegglen has been part of this project from its conception and her profound knowledge and experience with NETosis research, especially in animal models, were key throughout the process. From her lab also, Aaron Rogers and Gareth Mitchel played a fundamental role in training, data collection, sharing supplies, blood donation, confocal imaging, and helpful suggestions over the course of multiple trips to USU, zoom calls, and emails. Thank you for your generosity with your time and knowledge.

Finally, I'd like to thank the Utah State University Honors Program and those who support the Undergraduate Research and Creative Opportunities (URCO) Grant Program for supporting my endeavors to pursue undergraduate research and helping to prepare me for my career as I apply to future programs. These programs have significantly impacted and improved the quality of my educational experience here at USU.

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Introduction

The purpose of this project is to identify and characterize neutrophil extracellular traps (NETs) in the Naked Mole Rat (NMR) as a step towards understanding their remarkable cancer resistance. In doing so, we can translate this understanding to human cancer research in ways that could eventually inform new clinical treatments. Cancer is the second leading cause of death in the United States, exceeded only by heart disease. According to the Centers for Disease Control and Prevention, one of every five deaths in the United States is due to cancer. A growing area of research involves the analysis of cancer resistant traits in other species to understand their biological mechanisms and eventually make translations to human cancer research and clinical treatment. Because of their remarkable cancer resistance, NMRs are a prime subject for this research, and various studies have already suggested that the immune mechanisms of the NMR might be harnessed for human cancer therapies[1-4,7]. A background of the current relevant literature connecting these topics will first be given, followed by the project hypothesis, goals, and methodology section. A summary of our findings in the results section will be followed by a discussion of the project's potential significance for continuing research.

Although not the most common research model, NMRs have strong potential as a model for human immunology research. They are insensitive to low oxygen (hypoxia-tolerance), show no age-associated exponential increase in risk of dying and, most amazing of all, they are incredibly resistant to developing cancer[1-4]. NMR cancer resistance is manifested both in the absence of naturally occurring cancers and the lack of success in inducing tumors[2]. This contrasts sharply with many other animals, and especially other rodents. Mice show no such proclivity, with 50-90% of aged mice dying of cancer. In humans this number is 23%[4]. Studying cancer resistance in NMRs promises knowledge translatable to other mammals such as us.

Recently, researchers have begun to explore this avenue and have already discovered multiple cancer resistant traits. NMR cells show high tolerance of cellular stress and resistance to artificial tumor induction[3]. Specifically, NMR cells are hypersensitive to contact inhibition and so will stop growing at very low densities, thus arresting tumor growth. Another cancer resistance mechanism is the cellular production of high molecular weight hyaluronan which is believed to be an anti-inflammatory tumor suppressing extracellular polymer[6]. The longer and heavier NMR version of hyaluronan is more effective and more abundant than that produced by mice and humans[7]. An untested hypothesis has proposed that NMRs might also have additional tumor suppressor genes and have noted unique amino acid substitutions in cancer-related genes that could possibly change their function[2]. It is thus reasonably understood that NMR cancer resistance is multifaceted, with promising paths marked in multiple biological directions. Various studies have already suggested that the immune mechanisms of the NMR might be harnessed for human cancer therapies[1-4,7].

The cellular composition of the NMR immune system is markedly different from that of a mouse, a more common rodent research model[1]. NMRs have higher pro-inflammatory cytokine production in macrophages and different inflammatory responses to pathogens. In various immune cell proportions, the differences are staggering, and the ratio of myeloid to lymphoid cells is significantly higher *(Figure 1)*. NMR B-cells represent only one fourth of the amount found in

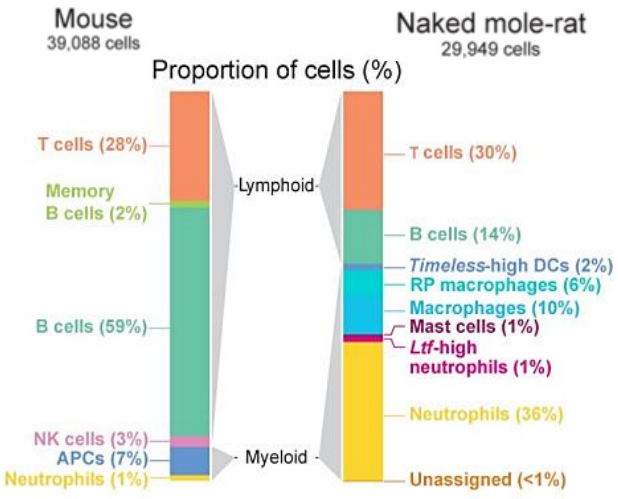


Fig 1*: scRNA-seq of mouse and NM-R spleens reveals major differences in immune cell populations, with proportions (%) labeled and each cell type color coded.* [1]

mice. Surprisingly, NMRs completely lack natural killer (NK) cells, a common type of tumor destroying cell. This in turn begs the question, by what mechanism do NMRs make up for and even exceed the anti-cancer activities of NK cells? One clue might be found in neutrophils, a key type of innate immune cell. NMRs have specialized neutrophils and possess a significantly higher percentage of neutrophils than mice. This ratio is similar to the neutrophil rich blood found in humans, though we do not share their cancer resistance. Is it possible that human and NMR NETosis are significantly different? Although this question has yet to be explored, current human neutrophil research has revealed additional layers of complexity that can help us know where to look.

Since their discovery in 2004, NETs have been studied mainly in mammals with specific focus on humans and mice. However, they have also been identified in cats, dogs, sheep, horses, elephants, and even some invertebrates[8]. NETs are a matrix of DNA and histone proteins that form outside of neutrophil cells. These extracellular webs function to trap and destroy microbes as part of the innate immune response[9]. *In vitro*, NETosis is stimulated by bacterial toxins including lipopolysaccharide (LPS) and ionomycin. LPS is a toxin produced by gram-negative bacteria that shows highly variable stimulation of NETosis[25]. Ionomycin is produced by a gram-positive bacterium and is a robust inducer of NETosis, yielding more consistent results than studies using LPS[25]. Although early research began to understand how NETs interacted with immune cells to stimulate differentiation and signal a proinflammatory response, increasing evidence indicates that NETs are a double-edged sword, and that high NET concentrations can have negative effects such as thrombosis, tissue damage, and autoimmune disease[8,10-12]. Most relevant to the current study, NETs are negatively associated with multiple stages of cancer development and progression in well-established, reputable research studies[5,9,13-20].

The biological relationship between NETs and cancer is complex and clinically relevant. The potential for NETosis-related mechanisms to yield therapies for cancer and other inflammatory diseases is well recognized, and a whole field is emerging to study this phenomenon[17,21]. Although the connection is clear, it is challenging to dissect the exact cancer-NET relationship because of interconnected immune response pathways. For example, an array of publications have identified that human cancer patients have elevated NET concentrations. [16,19,20]. Elevated NET concentrations have been identified in various types of cancer and in multiple types of tissue, from the tumors themselves to far reaching blood vessels.

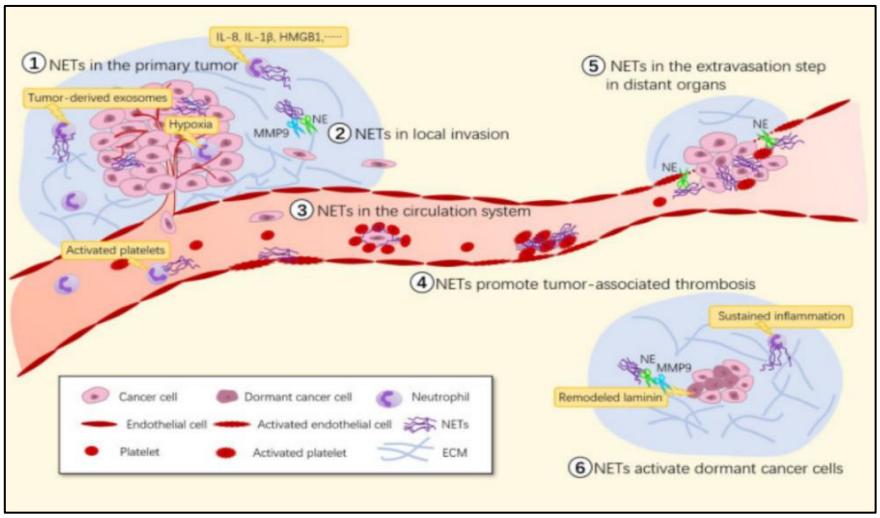


Figure 2: *Involvement of NETs in tumor growth and metastasis* [5]

Although neutrophils are immune cells, the NETs they produce have a multiplicity of cancer promoting effects, and cancer itself creates conditions that increase NET formation and activation *(Figure 2).*

Sustained inflammation and hypoxia around tumors have been shown to induce the activation of neutrophils to form NETs, which can then awaken dormant tumors[22]. Some specialists have proposed the value of NETs as biomarkers to help diagnose breast cancer early and predict cancer-related thrombosis[5,17]. A network of NETs settling in a blood vessel can act as a scaffold for cell coagulation, creating a blockage that can have devastating results[11,17]. New research has targeted the breakage of this scaffolding, which would greatly help improve patient

treatments[17]. The travel of NETs through blood vessels is also interconnected to cancer metastasis, with one study demonstrating that liver and lung NETs can even attract cancer cells from distant locations in the body[19]. When cancer cells are surrounded by NETs they are afforded a variety of benefits. NETs help improve tumor vascularity by creating space for blood vessels while simultaneously forming a physical barrier around tumors. The barrier shields tumors from the cytotoxicity of other immune responses, thus providing significant protection. This protection and blood supply markedly improve tumor growth, local invasion, and metastasis to other locations[13,15,16,19].

These results have come from studies involving both mouse models and human tissue samples, but the literature is void of any publications that have determined the character of NET release in NMRs. The aim of this study is to establish a protocol for the isolation, stimulation, and quantification of NMR NETosis to compare to human NETosis. It is hypothesized that NETosis is less active in NMRs than in humans, and thus that lower quantities of NETs will be measured in NMR neutrophils than in human neutrophils when treated with the same stimulus.

Methods

Gene sequence alignment:

An interspecies amino acid sequencing alignment was performed for NET component proteins using Benchling sequencing software. NCBI Reference Sequences for human, mouse, and NMR Citrullinated Histone 3 (H3) and Myeloperoxidase (MPO) were used to download DNA sequences into Benchling which were then translated into their respective amino acid sequences and aligned for comparison. The same antibodies for visualizing H3 and myeloperoxidase are used in both human and mouse experiments. By confirming that the NMR protein sequences for H3 and MPO are at least as similar to the human sequences as the mouse sequences, it was confirmed that the same primary antibodies could be used to visualize human and NMR H3 and MPO proteins *(Figure 3).*

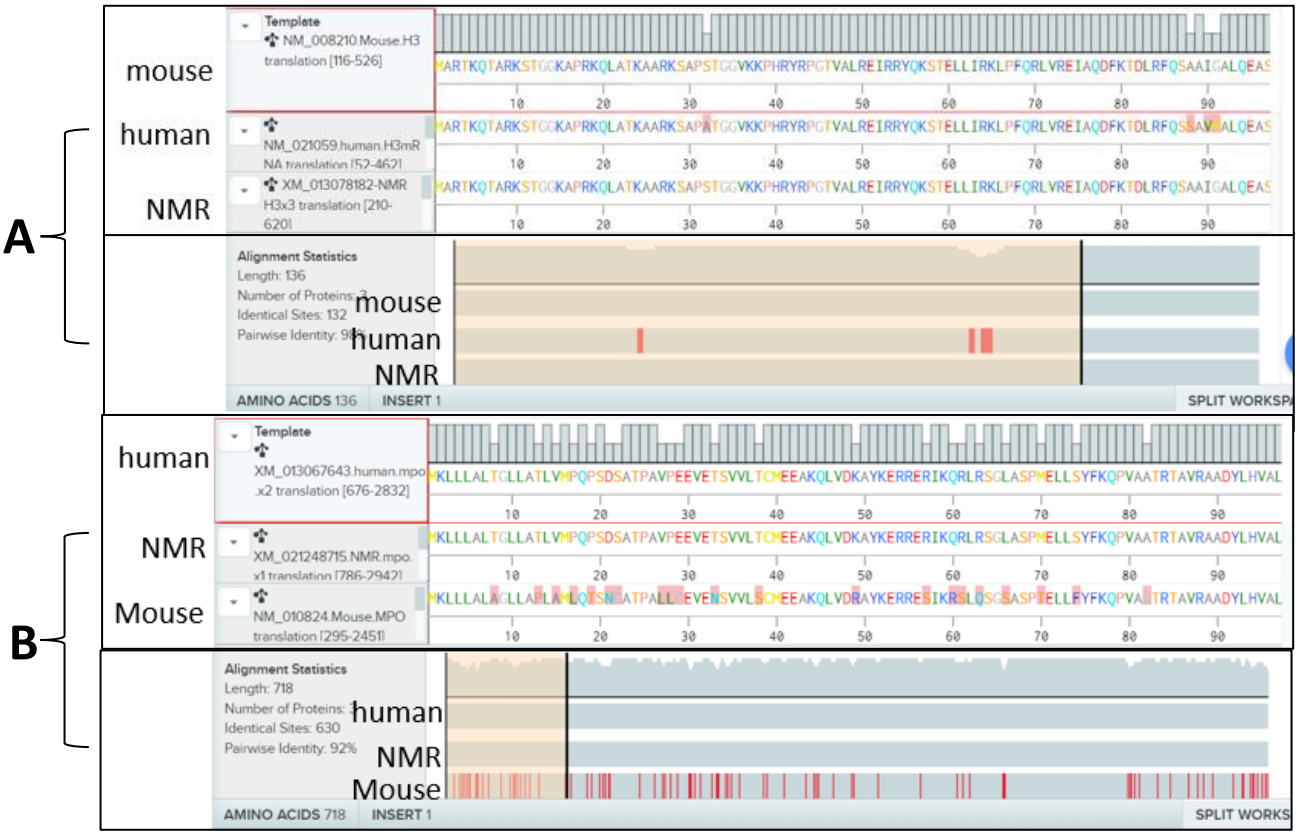


Figure 3. *Amino acid alignment between human, NMR, and mouse protein sequences for known NET protein components:* ***(A)*** *Citrullinated Histone 3 and* ***(B)*** *Myeloperoxidase.*

Sample Collection:

All NMR samples were collected from adult NMRs housed in Utah State University's Utah Science Technology and Research initiative (USTAR) BioInnovations Laboratory Animal Research Center (LARC). Venous blood was collected from animals according to an Institutional Animal Care and Use Committee (IACUC) approved protocol (#10053).

All human samples were provided by and handled only by researchers from the University of Utah Huntsman Cancer Institute in compliance with their tissue handling protocols.

Neutrophil Isolation[23]:

Blood was collected into EDTA- coated vacutainers and then centrifuged and allowed to settle in incubators set to body temperature (37°C for humans, 32°C for NMRs) for 30 minutes. plasma was collected from the top layer, diluted with PBS, suspended in Medium 199. Polymorphonuclear cells (PMNs; neutrophils) were isolated from the suspended plasma by purifying them from whole blood using a mono-poly resolving medium (MPRM) density

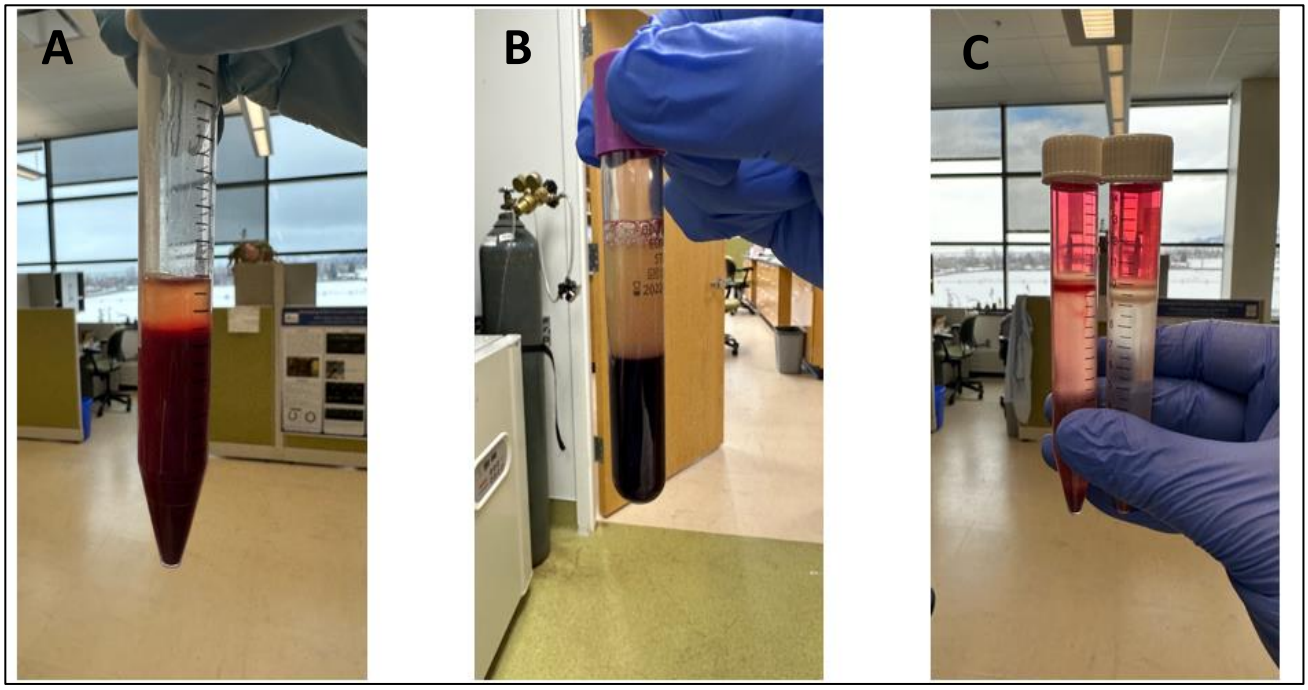


Figure 4. *Pictures showing blood separation.* ***(A)*** *NMR blood after being centrifuged for 10 minutes at 300g.* ***(B)*** *Human blood after incubating at 37 C for 30 minutes.* ***(C)*** *NMR (left) and human (right) density gradient separation after mono-poly resolving medium protocol, the bottom of the middle buffy coat contains the neutrophils.*

gradient protocol centrifugation for 30 minutes at 300 g with no brake. The lower buffy coat neutrophil layer was then collected, counted with a hemocytometer, and resuspended in PBS to a final concentration of 2 x 10^6 cells/ml (*Figure 4*).

Neutrophil stimulation and quantification with Sytox Green fluorescence assay[23]:

Cells were seeded into poly-L-Lysine coated plates, all samples except an unstimulated well of control neutrophils were treated with various concentrations of lipopolysaccharide (LPS) and ionomycin. Samples were then treated with a micro-nuclease enzyme to remove the extracellular DNA and transfer it into a black 96-well plate. It was then treated with Sytox Green, a high affinity nucleic acid stain that marks extracellular DNA that form NETs but will not cross cell membranes and mark intracellular DNA. A spectrophotometer was used to measure fluorescence signals of the Sytox stained NET DNA at an excitation wavelength of 485 nm and emission wavelength of 523. Triplicate measurements were recorded of the fluorescence intensities for each well to quantify the NETosis of each sample and compiled into tables. This data was normalized based on the average of triplicate Sytox measurements from each samples control of unstimulated neutrophils, and then imported for statistical analysis and graphing with Prism 9 software. A two-way analysis of variance was completed comparing ionomycin induced NETosis in human and NMR neutrophils across multiple dosages. A t-test was used to compare the lowest dose ionomycin NET stimulation to the unstimulated sample for each species to confirm that the level of induced NETosis was statistically significant for both human and NMR neutrophils.

Confocal Microscopy Fluorescence Imaging[11]:

Unstimulated neutrophils were added to poly-L-lysine coated 8-well chamber slides and allowed to adhere for 60 minutes. They were then fixed with a 4% formaldehyde solution,

washed with PBS, permeabilized with 0.5% Triton, and washed with PBS again. Animal-Free Blocker and Diluent were added for 60 minutes at room temperature, before primary antibodies (anti-Histone 3 and anti-MPO) and secondary antibodies (AF488 and AF650) were sequentially added and allowed to incubate for 60 minutes each at room temperature before being washed with a tris-buffered saline polysorbate solution (TBST). A drop of DAPI mixture was carefully placed onto each slide and allowed to cure for at least 4 hours in the dark. The slides were then sealed with nail polish and ready to be observed with a laser confocal scanning microscope**.** Images were captured of both unstimulated neutrophils (Figure 5 A-C) and stimulation induced NETosis (Figure 5 D-F), with different colors showing different structures.

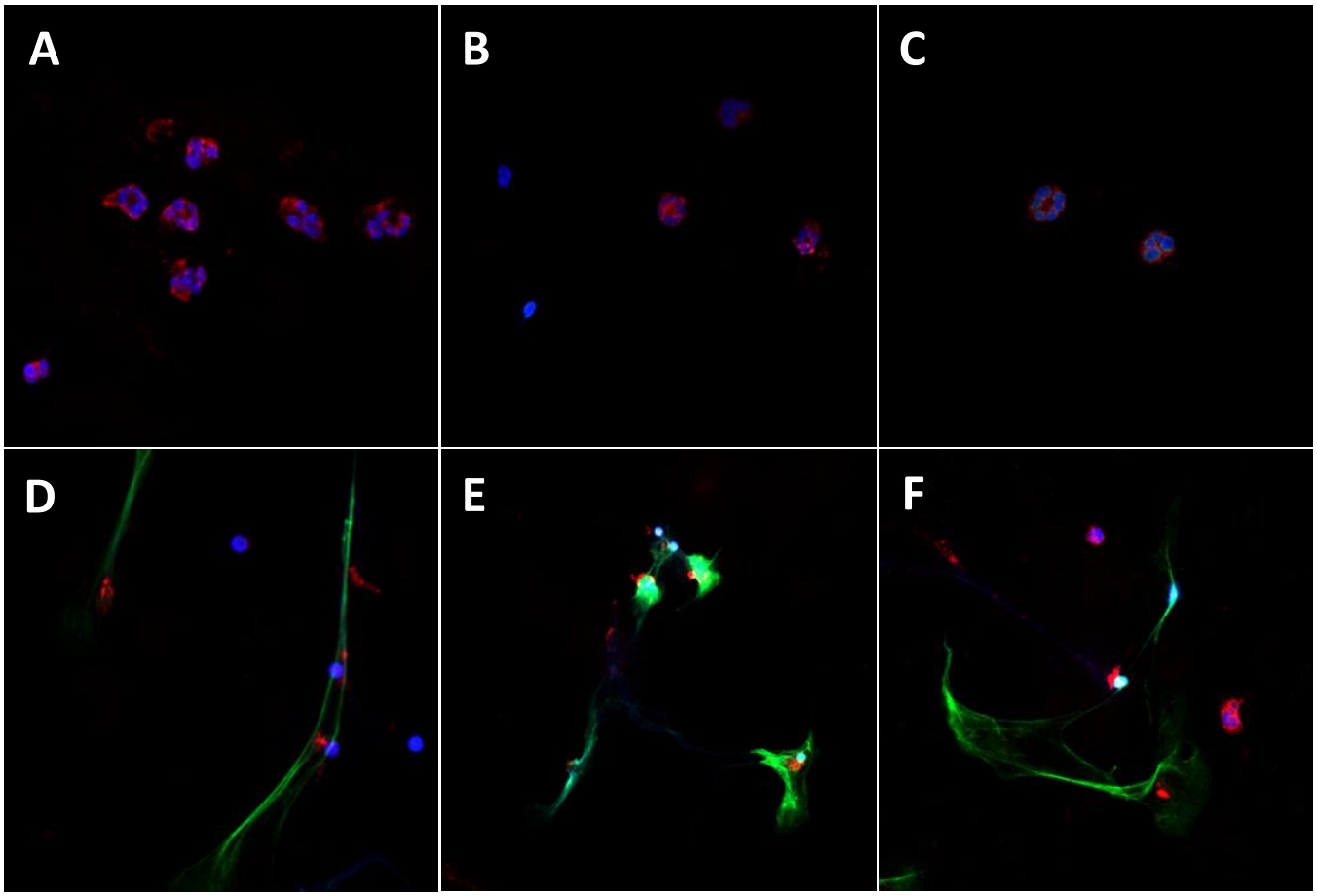
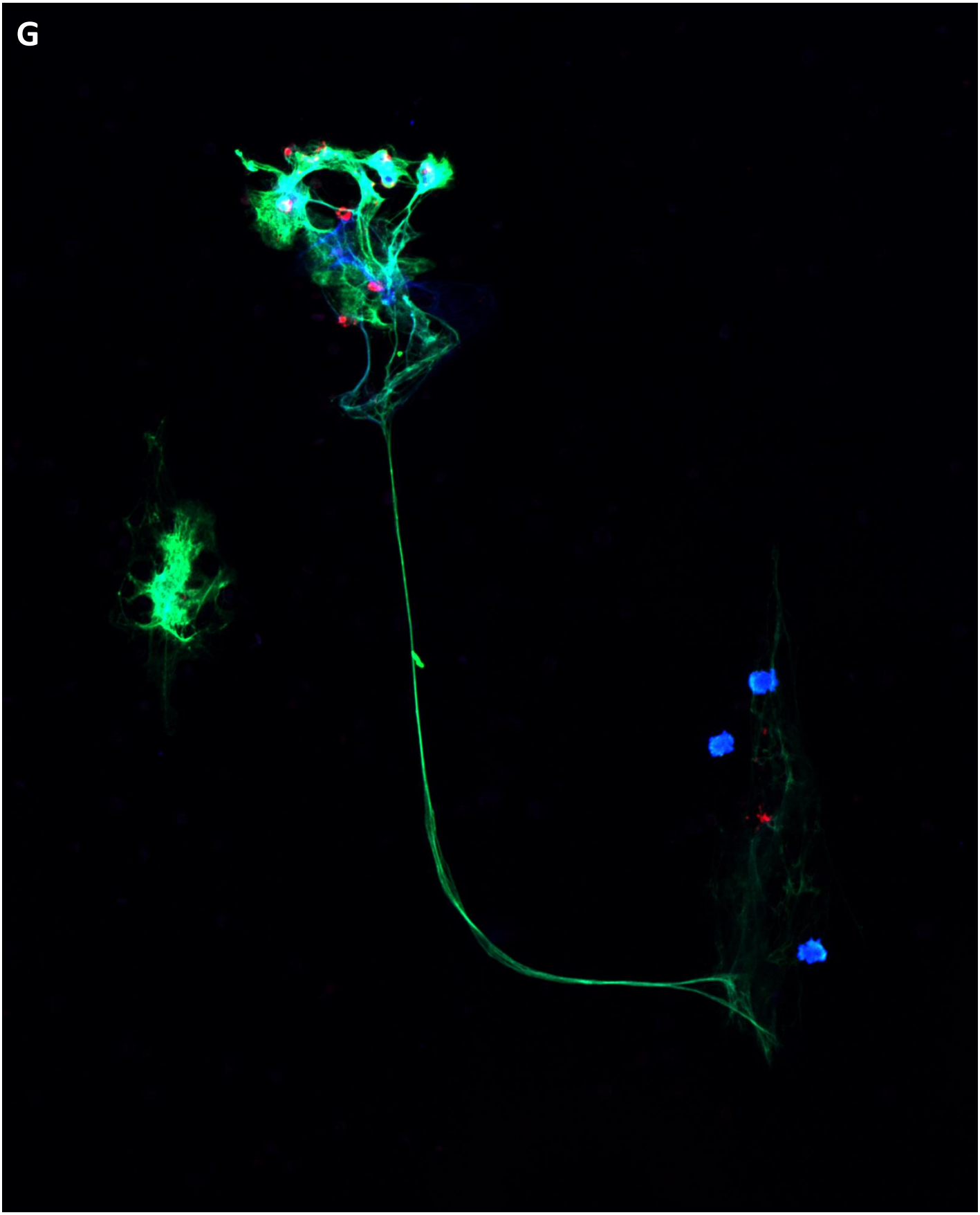


Figure 5*: NMR neutrophils detected via confocal microscopy. Colors indicate anti-Histone H3 (green), H/M Myeloperoxidase (red) primary antibodies. Nuclei stained with DAPI-conjugated mountant (blue).*
(A-C) *Untreated NMR neutrophils showing typical multi-lobed nuclei.* ***(D)*** *2 uM ionomycin stimulated NET.* ***(E)*** *10 uM ionomycin stimulated NET.* ***(F)*** *100 ng LPS stimulated NETosis at various stages.* ***(G; next page)*** *Dramatic NMR NET stimulated with 2uM ionomycin.*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Results

We successfully developed a protocol for the isolation of NMR neutrophils and stimulation of NETosis. To our knowledge, this is the first study of NETosis in NMRs, and the first ever images of NMR NETs. After treatment with bacterial stimulants ionomycin and LPS, microscope visualization showed that most NMR neutrophils were still whole, in contrast to most of the human neutrophils which had already burst and released their NETs. Sytox green assays quantifying the extracellular DNA of NETs showed significant differences between human and NMR NETosis *(Figure 7).* After all data was normalized to control proportions, two-way ANOVAs revealed a main effect of species for both the ionomycin treatments ($F_{1,26}$=1006; $p<0.0001$) and LPS treatments ($F_{1,28}$=37.99; $p<0.0001$). All human ionomycin samples were statistically different than their control, while only the highest ionomycin dosages were statistically different in NMR samples. This indicates that higher dosages of ionomycin treatment were required to induce significant NETosis in NMR neutrophils compared to human neutrophils. Differences from LPS-stimulated NETosis were significant between species, but not across concentrations, though LPS is reported to be a less reliable NET stimulant that produces variable results[25]. All data is a composite of triplicate measurements, and multiple iterations of ionomycin NET stimulation showed similar trends: human neutrophils released more NETs than NMR neutrophils. This surprising outcome indicates deceased NETosis in NMRs when compared to humans.

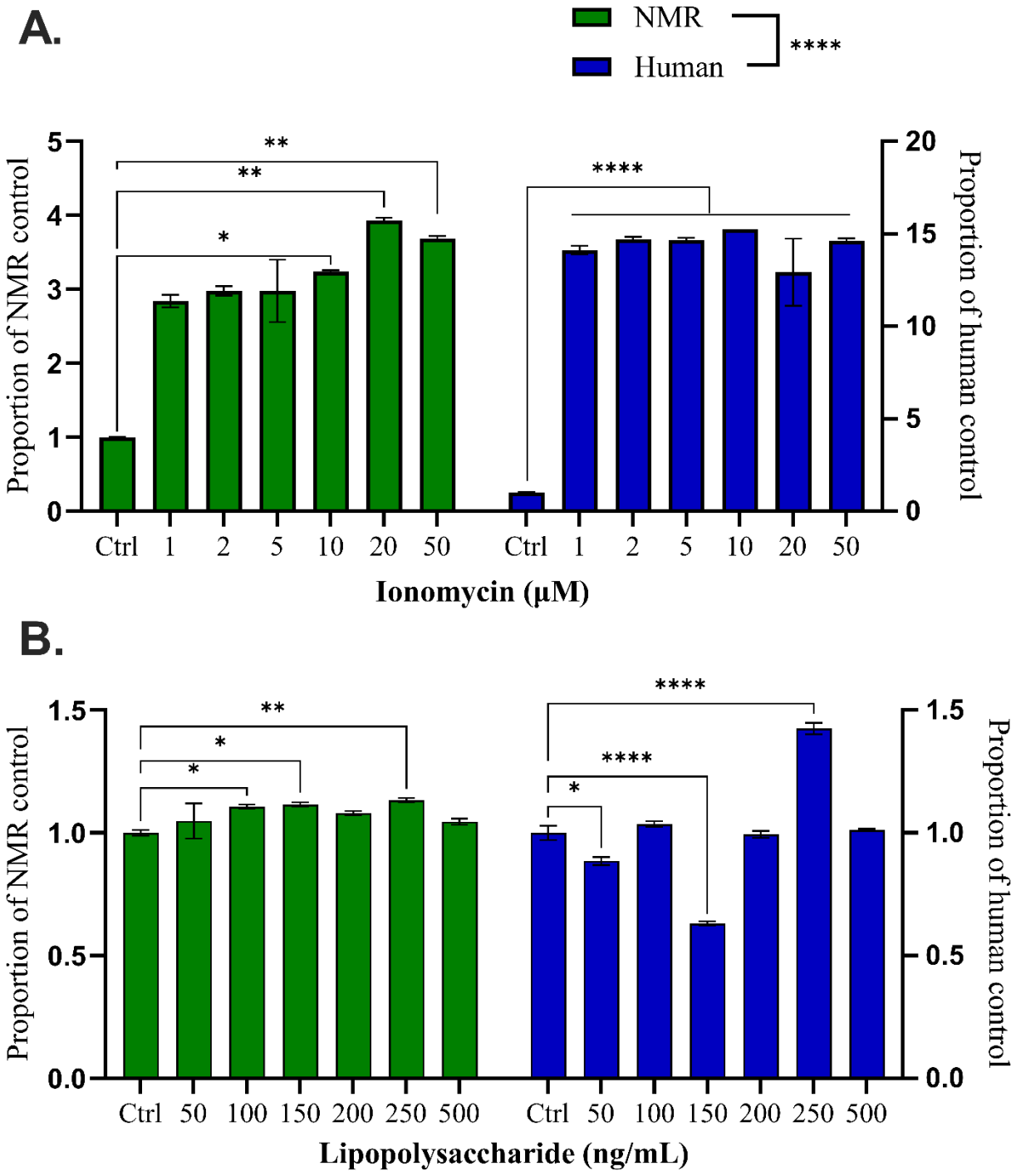

***Figure** 7. Sytox Green Assay data analysis of control normalized data quantifying NETosis reveals a main effect of species for both treatments. **(A)** ionomycin induced NETosis showed statistical differences between dosages that indicate higher resistance to induction of NETosis in NMRs. **(B)** LPS induced NETosis results in human and NMR neutrophils were inconsistent.*

Discussion

Characterizing NETosis in NMRs is an important study that fills a gap in the literature. While NET research has been done with humans, mice, and other mammals, this is the first time NETs have been characterized in NMRs. Because of the significant role played by NETs in cancer, our findings point to the possibility that less active NETosis in NMRs may be one of the underlying mechanisms contributing to their cancer resistance. Although more sampling will be necessary to further confirm and quantify these differences, this initial success nonetheless helps to confirm the value of researching NMR NETosis and shows promise for future experiments.

NMRs possess an incredibly unique cancer resistant phenotype and do not develop tumors, which makes them an invaluable resource, and the potential of the NMR as a cancer resistance model is well recognized. The next steps are to continue replicating these experiments and to work to understand the mechanisms of NET release in NMRs. It is possible that NMR neutrophils have differences in their cell membranes that make them less permeable to toxins. Another possibility is that they have different concentrations of intracellular ions or enzymes, or different enzyme structures, that impede signal transduction cascade amplification, effectively dampening chemical signals that in humans would be much stronger. If cancer therapy drugs in humans could inhibit neutrophils to imitate the less active NETosis of NMR neutrophils in a controlled manner, it might be possible to significantly reduce their cancer-inducing effects. Targeted NET inhibition could drastically change the extracellular landscape. The effectiveness of natural cancer killing immune cells and current cancer therapy drugs could be increased by removing the protective shield that NETs form around cancerous cells, perhaps decreasing the negative side effects of the toxic dosage required to kill a tumor. Reduced NET concentrations could mitigate NET activation of tumor cells and increased tumor vascularization, thus lessening cancer cell activity and tumor growth. It is even possible that we could rein in the contribution of

NETs to cancer cell metastasis. Multiple mammals possess effective ways to combat cancer, and a deeper understanding of these mechanisms will likely lead to significant medical breakthroughs in cancer treatment. NET research will play a key role in these developments. As one article reviewing current knowledge so powerfully states "We propose that the rapid developments in the field of NETosis may provide new targets to combat the consequences of cancer and perhaps even help to contain the disease itself" [17]. Many researchers have observed that a better understanding of the NET-cancer relationship can help with early diagnosis, prevention of thrombosis, cessation of metastasis, and more effective killing of tumors [5,17]. This knowledge also holds significance in breakthroughs that could be made for a host of other diseases and infections where NETs sometimes harm more than they help including: heart disease, stroke, COVID-19, inflammatory or autoimmune diseases such as asthma, rheumatoid arthritis, celiac disease, multiple sclerosis, lupus, HIV/AIDS, and countless others[26.] Neutrophils account for up to 70% of human immune cells, [24] and we are only beginning to understand the role of NETs in disease progression. An increased understanding of the pathways of NETosis will help us know how to fortify our immune system and prevent cellular treason. This first study of NMR NET release helps to light the path and brings us one step closer towards beating cancer ourselves.

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Reflective Writing

My honors capstone research project has been more work than I ever anticipated. The difficulty of the project was increased because of the subject matter of my research. Neutrophil extracellular traps had never been researched in naked mole rats, and to our knowledge no one at Utah State University is doing research about NETs. There is no human medical research on campus, and so I did not think that I would be able to ever be involved with work that would relate so directly to my goal of becoming a physician. The process all began because I was attending ADVS weekly seminars with visiting speakers. I went to the lecture of Dr. Con Yost and heard his presentation about the contribution of NETs to COVID. After his lecture I went up and spoke to him, I gathered his contact information, and scheduled a time to shadow him. He works at Primary Children's Hospital in Salt Lake City as a neonatologist. Shadowing him was a life changing experience. I had never been in an intensive care unit at a hospital, nevertheless seen premature infants on life support. I have wanted to become a physician for years, and have even shadowed many other doctors, but those children touched me in a way that deepened my passion to help like nothing else. I had no idea that attending that Animal Dairy and Veterinary Sciences seminar series would make a significant impact on my career motivation and lead me to my capstone project.

When I spoke to Dr. Yost about his research and I expressed interest in his work, he connected me to one of his colleagues, Dr. Lisa Abegglen, who works at the Huntsman Cancer Institute at the University of Utah medical school. She and my mentor Dr. Wang had a call to talk about the potential of collaborating on a research project. I soon realized that interdisciplinary teams are common in advanced research, and I am so grateful for the opportunity that I have had to collaborate with so many amazing people with different backgrounds and skillsets. My lab

work with Dr. Wong has involved using rodents as models for human immune system research. Dr. Wang has one of the few naked mole-rat colonies in the United States, and so Lisa was thrilled at the idea of us doing a collaboration to understand NETs in naked mole rats. She had been researching them in elephants, mice, humans, and other animals but nobody had ever looked at them in naked mole-rats, and she was thrilled by the prospect! I was able to learn more about professional collaboration and teambuilding as I scheduled zoom calls, coordinated training, visited her lab, and welcomed researchers visiting our lab.

This project ended up being the perfect culmination of my whole undergraduate career. Because I majored in biology with a minor in chemistry I took many courses learning about biochemistry, cellular biology, evolution, anatomy, and physiology. This was a combination of them all. The lab courses I have taken involved designing experiments, collecting data, and statistical analysis, however, I had never completed something of this magnitude before. My work ended up bridging the gap that I have always felt between my degree and my career. This would not have been possible without the remarkable networking of Dr. Wang. Dr. Wang was enthusiastic about the idea of a collaboration, and he worked to make this project possible for me.

Dr. Wang has been an incredible mentor was excited to help me learn about and experience the professional research process. He encouraged me to apply for the undergraduate research and creative opportunities (URCO) award, which I didn't even know existed. The URCO application was a rigorous affair and included writing a lengthy proposal, the first one that I have ever written outside of a class. I was intimidated to go so far out of my comfort zone and the experience of my major, but thanks to him I applied for and received this award. The initial process of writing was extremely challenging, as writing has never been my strength and I

have never previously had to do such an in-depth literature review. I was astounded by the complexity of the material as I delved deeper and deeper, finding connections that were totally new to me! I also found out as I was writing it that formatting and citations are a major challenge in and of themselves! This was also the first time that I have participated in presenting at a research symposium, and I found that I thoroughly enjoyed not only sharing what I have learned, but also connecting with the passionate minds of researchers from the myriad a disciplines that were represented at the Student Research Symposium. I have gained a profound appreciation for the scientific process and those who dedicate their lives to research.

Engaging in a broader community of researchers at multiple institutions working has helped me to understand the price that they pay to obtain the knowledge that is changing the future. I was amazed learning about their projects, and inspired to think that the work that I am doing now could potentially contribute to something greater: the ability to truly help those who are suffering. Although I love to learn I have never really considered myself a research scientist. Now however, thinking about the promising potential of naked mole-rat neutrophil extracellular trap cancer research has convinced me to continue working in Dr. Wangs lab as a research assistant for an additional year after my graduation this spring. I hope to find out more about naked mole-rat cancer resistance and continue collaborating with Dr. Abegglen to learn more about ongoing human neutrophil extracellular trap cancer studies.

I truly feel that my capstone project not only has been the pinnacle of my undergraduate experience, but also has formed a launch pad to help me transition into my future. I already feel that my experience will aid me as I apply to medical schools this upcoming cycle. Due to the amazing experience that I have had during this project, I may even consider participating in medical research during medical school and throughout my career, which I had never previously

considered. Dr Wang is encouraging me to investigate MD-PhD programs and has helped me to see the doors that immunology researchers are opening for modern medicine. I am sure that the value of my current experiences will continue to contribute to my future, and I only wish that I had started earlier! As I finish this project I feel that I need to tell other undergraduate students (especially a younger version of myself) that there are many opportunities for growth at Utah State University that are outside of the classroom. Participating in research can add meaning to the required courses that sometimes seem so disconnected from your goals. Take advantage of the doors that the Honors Program will open for you, and don't be afraid to stretch outside what you think is your lane. I have accomplished more than I thought that I could, and you can do. Have the courage to use your own reason and *Sapere Aude.*

(Word Count 1192)

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NETosis in Naked Mole-Rats: *a step towards cancer resistance*

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Introduction

Neutrophils are the most abundant immune cell in humans**.** They release *neutrophil extracellular traps* (NETs), a process referred to as NETosis, as part of an immune response to trap diseases. Unfortunately, NETs also worsen the effects of many diseases. **NETs significantly contribute to the growth and metastasis of cancer** (1).

***Naked mole-rats* (NMRs) are extremely cancer resistant**, but still have a high percentage of neutrophils in their immune system (2). **Studying how their neutrophils and NETs are different than ours could lead to better cancer treatments and increased survival.**

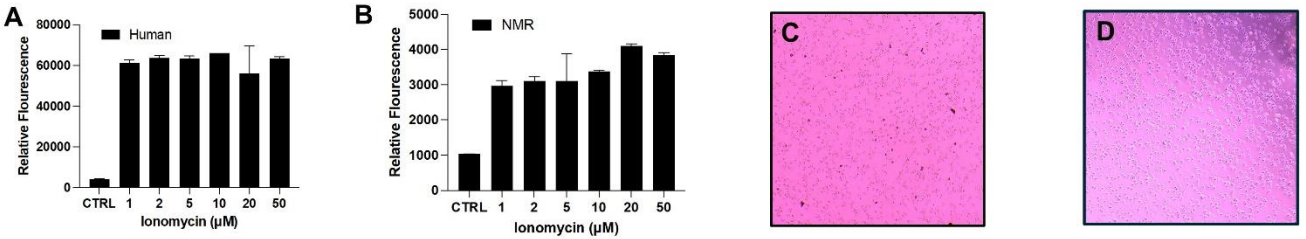
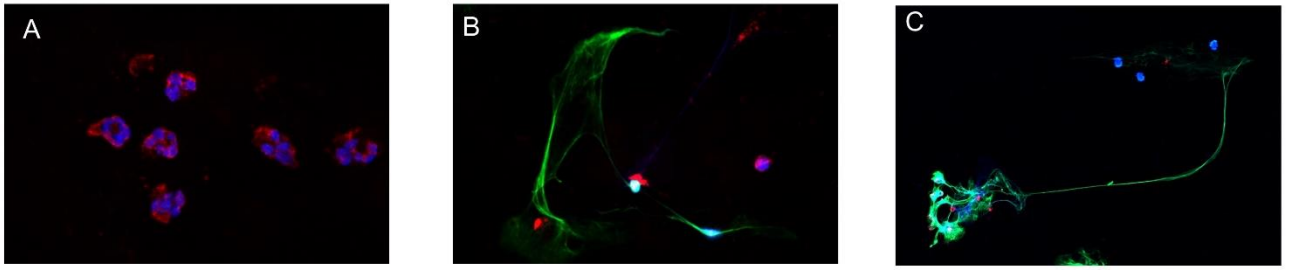


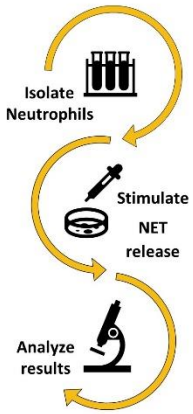
Figure 1. (A) Human NET release with ionomycin was quantified with CYTOX Green assay. **(B.)** NMR NET release with ionomycin was quantified with CYTOX Green assay. NETosis. **(C.)** Bright light image with 50 uM ionomycin treatment in human neutrophils **(D.)** Bright light image with 50 uM ionomycin treatment in NMR neutrophils



Figures 2. Confocal microscopy images. Each color binds different cell structures for visualization. (BLUE: Nuclear DNA, RED: Myeloperoxidase, Green: Histone H3.) **(A.)** Unstimulated NMR neutrophils with typical multi-lobed nucleus. **(B. & C.)** Ionomycin Stimulated NMR neutrophils at different stages of NET release.

Methods

NMR blood was collected and then separated via cell sedimentation and density gradients to isolate neutrophils. Cells were counted and seeded onto plates for the stimulation of NETosis with the bacterial toxins ionomycin and lipopolysaccharide (LPS). Results were collected via a CYTOX Green fluorescence assay and confocal microscopy (3).



Conclusion & Significance

Protocols were successfully established for neutrophil isolation and induction of NET release, and NMR NETosis was characterized for the first time. Preliminary data showed that compared to NET releases in human neutrophils, notably higher stimulation dosages are needed to stimulate NMR neutrophils to release NETs, indicating that NETosis may be a less active process in the NMR immune system. This feature would surely contribute to their lack of cancer.

Our study has shed light into the relationship between NETosis and cancer resistance in the NMR. Future research will continue to clarify NET disease involvement pathways and may eventually translate these findings to the development of new cancer therapies and better outcomes.

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Appendix: Spring 2023 USU Undergraduate Student Research Symposium Poster

Author Biography

Thomas Abraham Smith was born and raised in Corcoran Minnesota, a small-town northwest of Minneapolis. He currently resides in Logan Utah with his wife Jessi of three years, who he met in a USU Spanish class, and together they are expecting their first child. During his undergraduate career, Thomas has participated in the Honors Program, served as a Social Action Lead for the Val R. Christensen Service Center, taught lab sections as a teacher's aide, sung in USU Chamber Singers, and worked as a research assistant. He has been the recipient of multiple scholarships, including the Undergraduate Research and Creative Opportunities award, and is regularly on the Dean's list. Thomas is a senior graduating with a Bachelors of the Arts in Biology, with an emphasis in Human Biology and minors in Chemistry and Spanish. He is currently preparing to take the MCAT and will be applying to medical school this summer.