THE REDUCTION OF NEUTROPHIL POPULATIONS IN INFLAMMATORY CONDITIONS USING CD177-MEDIATED PH-SENSITIVE FUSOGENIC NANOPARTICLES

by

Esther Suikhinpar Thang

B.S (Salisbury University) 2013

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

August 2019

Accepted:

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Craig Laufer, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
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Senta Kapnick, Ph.D. Committee Member	
_____	_____
Rebecca Erwin-Cohen, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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DEDICATION

This is dedicated to my family. For without their patience and support, none of this would have been possible.

ACKNOWLEDGEMENTS

Thank you to the reading committee Drs. Rebecca Erwin-Cohen, Craig Laufer, and Senta Kapnick. I would also like to thank the rest of the graduate faculty at Hood College for making this degree so enjoyable to obtain.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
The reduction of neutrophils population in the inflammatory condition using cd177-mediated pH-sensitive fusogenic nanoparticles.

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
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3c. POSITION TITLE
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3f. MAJOR SUBDIVISION
Biomedical Science Program

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4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	
☒ No ☐ Yes	N/A	
4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☐ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name: Esther Suikhinpar Thang
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10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE
08/7/19

Face Page

PROJECT SUMMARY (See instructions):

Neutrophils serve a vital role in innate immune defense against bacterial and fungal pathogens. While neutrophils function in the most robust immune defense, they can also play a detrimental role in inflammatory conditions that can even lead to death. Reduction of complement factor 5a receptor-1 (C5aR1) suppresses neutrophils to get signals to migrate to the affected site, thus reducing inflammation. Previous research conducted by Miettinen et al. in 2018 demonstrated that 67-82% of mouse neutrophil C5aR1 protein was knocked down by siRNA and antisense oligonucleotides (ASO) using CD177-mediated hybrid polymerized liposome nanoparticles (HPLN). Approximately 30% of HPLNs were degraded in the endosome. In this proposal, I will incorporate a pH sensitive Glutamic acid-Alanine-Leucine-Alanine (GALA) peptide on the surface of HPLN, which will help the HPLN escape the endosome. I hypothesize this will completely and transiently knock down neutrophil C5aR1.

RELEVANCE (See instructions):

Neutrophils play an important role not only in innate immune reactions, but also in a wide variety of other functions. Regulation of some of these functions through specific targeting of neutrophils, or a subpopulation of neutrophils, would be therapeutically advantageous in inflammatory diseases.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Esther Suikhinpar Thang	B.S
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Salisbury University	B.S	2013	Medical Laboratory Science
Hood College, Frederick, MD	M.S.	2019 (anticipated)	Biomedical Science

A. Positions and Honors

B. Selected peer-reviewed publications (in chronological order)

C. Research Support

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Clinical:

N/A

Animal:

N/A

Computer:

Office:

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

•Centrifuges - For collection of cell lysates
•Thermal cyclers - DNA amplification and RT-PCR
•
•
•
•

The following consumables will be purchased with grant funding:

• Petri Dishes
• Immunofluorescence microscope
• Becton-Dickinson Accuri C6 Flowcytometer
• Electron Microscope, J-725 K (JASCO, Japan)
• LMS 5 PASCAL laser scanning confocol micropscope
• Antibodies
•
•
•
•

SPECIFIC AIMS

Specific Aim 1: Incorporate fusogenic Glutamic acid-Alanine-Leucine-Alanine (GALA) peptides on CD177 mediated Hybrid Polymerized Liposomal Nanoparticle (HPLN)

The GALA peptide can be attached on the outer surface of the HPLN in a manner similar to which human CD177-peptide binding was incorporated in the HPLN (Miettinen 2018). HPLNs with surface displayed peptides, CD177-peptide binding, and GALA peptide will be prepared by Nano Valent Pharmaceutical, Inc. The incorporation of GALA peptide on HPLN will be examined using Circular Dichroism Spectra Analysis.

Specific Aim 2: Examine the binding, internalization, specificity, and endosomal escape potential of the GALA-HPLNs.

Chinese Hamster Ovary (CHO) cells expressing human or mouse CD177, and human or mouse blood-derived neutrophils will be used to observe the internalization, localization, specificity, and ability to escape the endosome. Techniques employed will include immunofluorescence microscopy, confocal laser scanning microscopy, and flow cytometry.

Specific Aim 3: Examine the knockdown of human C5aR1 protein using anti-sense oligonucleotides (ASO).

The life span of neutrophils *in vitro* is about 8 hr (Pillay 2010) which would be too short to sustain for a knockdown experiment. The knockdown delivery by ASO is expected to occur between 4-72 hours after administration (Miettinen 2018). CHO cells express various combination of human or mouse C5aR1, C5aR1-GFP, and CD177 will be used for the experiment. The third generation LNA GapmmeR^{TM} ASOs containing a

phosphorothioate backbone and locked nucleotides in each end will be used (Chan 2006). The protein knockdown will be measured by flow cytometry and mRNA knockdown will be measured by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

BACKGROUND AND SIGNIFICANCE

Introduction

Inflammatory Response

Inflammation is a vital part of the human immune system. It is known as a complex cascade of events that is induced by the response to infection or tissue injury. Inflammation can be either an acute or chronic. Short term effects of inflammation enable a person to fight the infection, clear up the dead cells or debris, and heal the wound (Chen 2017). However, long-term inflammation effects can be problematic and include the unresolved inflammation that causes chronic inflammatory diseases such as arthritis, autoimmune diseases, inflammatory bowel diseases, cardiovascular disease, and Type-2 diabetes (Chen 2017).

The symptoms of inflammation are described as redness, swelling, pain, and loss of tissue function (Punchard 2004). These symptoms are the result of an increase in vasodilation that causes an increase in blood volume in the affected area. The increase of blood volume produces pyrexia in the local tissue environment and can be visualized as reddened skin or tissue (Punchard 2004). The permeability of the vascular spaces also permits the leakage of fluid from blood vessels which result in the accumulation of fluids (edema) that produces swelling in the tissues. These activities promote and enhance the entrance of white blood cells (WBC) into tissues from the local capillaries. The inflammatory response also activates resident tissue cells such as macrophages, mast cells, and dendritic cells (Michels da Silva 2019). These cells release chemokines, cytokines, and other soluble mediators to recruit more WBC and other phagocytic cells.

Once WBC have arrived at the infected site, they are activated to phagocytize bacteria and debris and produce more mediators to amplify the response.

Neutrophils are the first to be recruited to the affected site where they amplify local innate responses (Selders 2017). They play a major role in the inflammatory response, antigen presentation to T-cells, and release cytokines and chemokines to recruit other phagocytic antigen presenting cells, such as monocytes and dendritic cells (Selders 2017).

The resolution of the inflammatory response is critical to prevent unnecessary "bystander" damage to tissues (Jorch 2017). If the inflammation is not terminated completely when it is no longer needed, there can be serious consequences that cause cellular destruction, chronic inflammation, and death (Kloner 1993). The inflammation is terminated when the infection is removed, and healing of the affected tissues begins (Michels da Silva 2019). The recruitment of neutrophils is deactivated, and the apoptotic process is initiated (Michels da Silva 2009). The apoptotic neutrophils are phagocytosed by macrophages leading to neutrophil clearance. Consequently, the anti-inflammatory and reparative cytokines, such Transforming Growth-Factor β1 (TGFβ-1), are released (Yoshimura 2010). Once the healing process begins, macrophages enter the lymphatic system for apoptosis or process the antigen to establish a memory response (Yoshimura 2010).

Unresolved inflammation can result from the inability to clear the infectious organism (e.g., *Mycobacterium tuberculosis*, protozoa, fungi, and other parasites), exposure to certain irritants or foreign materials that can escape the enzymatic breakdown or phagocytosis, autoimmune disorders (e.g., Rheumatoid arthritis), recurrent episodes of

acute inflammation (e.g., tuberculosis), and biochemical inducers that cause oxidative stress or mitochondrial dysfunction (Holers 2018). The ability to control such unwanted inflammation is critical in the survival of cells and for the prevention of further damage to affected tissues (Chan 2017).

The World Health Organization (WHO) ranks chronic disease as the greatest threat to human health (WHO 2009). In a report by the Rand Corporation in 2014, nearly 60% of Americans had at least one chronic condition, 42% had more than one, and 12% of adults had five or more chronic conditions (Buttorff 2017). The prevalence of chronic diseases associated with chronic inflammation is anticipated to increase persistently for the next 30 years in the United States (Buttorff 2017). Chronic inflammation-mediated disease such as diabetes, cardiovascular diseases, arthritis, allergies, and chorionic obstructive pulmonary disease have taken millions of lives each year (Peter 2016). It is critical to continue research to develop management protocols for these diseases (Linlin 2018).

Inflammation is also a critical component of tumor progression (Coussens 2002). Many cancers have been associated with infection, chronic irritation, and inflammation (Coussens 2002). Persistent infections within the host induce chronic inflammation. Phagocytic cells such as neutrophils produce reactive oxygen and reactive nitrogen species that are used as part of the fight against infection. Such elements can form peroxynitrite, a mutagenic agent (Kiraly 2015). Repeated tissue damage and regeneration of new tissue in the presence of highly reactive nitrogen and oxygen elements can result in DNA undergoing permanent genomic alterations such as point mutations, deletions, insertions, or rearrangements (Jackson 2009). It has been confirmed that chronic

ulcerative colitis and Crohn's disease are the main drivers of colon carcinogenesis (Chang 2018). Infection with *Helicobacter pylori* is the leading cause of stomach cancer (Wrolewski 2010). Neutrophil activation can increase the impact of myocardial infarction after reperfusion (Niederbichler 2006). The ability to suppress acute inflammation is also crucial in preserving the health of affected tissues and preventing further damaged in specific illnesses such as ischemia reperfusion injury, neurodegenerative diseases, and sepsis (Chen 2017).

Neutrophils

Neutrophils are the most abundant granulocytes and the most abundant type of white blood cells in most mammals (Mócsai 2013). They serve a vital role in innate immune defense against bacterial and fungal pathogens. As neutrophil are the first line in defense of foreign pathogens, their capability to rapidly and robustly kill invaders is indispensable. The role of neutrophils in mediating immune responses against infectious microorganisms, tissue restoration, protease regulation, and vasodilation is vital, yet neutrophils often cause significant collateral damage to the host (Jorch 2017). The neutrophil role in inappropriate inflammatory conditions such as myocardial infarction, sepsis, and neurodegenerative disease can be detrimental. The window to maintain and to displace the active neutrophil in the local tissue is critical to both defending the host, but also to maintain safety of the surrounding tissues. It has been found that their ability to migrate quickly to damaged tissues can have a pro-metastatic role in the spread of cancer cells (Uribe-Querol 2015).

Previous studies established that Complement Factor 5a (C5a) binds to Complement Factor 5a Receptor-1 (C5aR1) on neutrophils. These receptors are one of

the primary chemoattractants that are involved in neutrophil-mediated injury that is associated with myocardial infarction, sepsis, and neurodegenerative diseases (Wright 2013).

Neutrophil Development

Neutrophils are born from pluripotential hematopoietic stem cells in the bone marrow. Neutrophils usually spend their life span in three different compartments; bone marrow, blood, and tissues. Neutrophils proliferate, differentiate, and mature in the bone marrow which takes about 10 d (Postnikoff 2017). Then, they are released in the peripheral blood and stay there for about 5-9 hr (Postnikoff 2017). Once activated, the tethered neutrophils migrate to the designated tissues to perform their function of host defense. After completing their missions, they die at the afflicted site which normally takes about 1-2 d (Soehnlein 2008). Neutrophils that do not receive the recruiting signal generally go through apoptosis (Bratton 2011).

Neutrophil production and maturation are primarily regulated by Interleukins-3 (IL-3), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), and Granulocyte Colony Stimulating Factor (G-CSF) (Cetean 2015). During the process of maturation, neutrophils go through six stages that can be morphologically distinguished (Choi 2017). The stages are myeloblast, promyelocyte, myelocyte, metamyelocyte, non-segmented neutrophil, and finally to segmented neutrophil; the mature and readily activatable neutrophil. The progression of morphological changes occurs in the nucleus and in the cytoplasm. The nucleoli disappear, the chromatin condenses, and the nucleus eventually become segmented, hence the name segmented neutrophils. In the mature neutrophils, the cytoplasm becomes pink-to-tan color and contain various granules. These granules

contain the proteins necessary to mediate the recruitment, chemotaxis, antimicrobial function, and extracellular trap formation of neutrophils (Choi 2017). There are four granules that are sequentially deployed as neutrophils arrive at infected sites. First, secretory vesicles are released to enable the recruitment of more neutrophils from the blood. Then, specific and gelatinase granules are released to enable neutrophil migration and begin the formation of an antimicrobial environment. Finally, azurophilic granules release potent antimicrobial proteins at the site of infection and into phagosomes (Tang 2017).

About half of the total of the mature neutrophils are freely circulating (circulation pool) in the blood vessel while the other half are tethered (margination pool) along the blood vessel walls by a loose binding interaction of the selectin adhesions. The two populations are in equilibrium and can be rapidly exchanged. Once activated, the tethered neutrophils migrate to the designated tissues by diapedesis to perform their function of host defense. After completing their missions, they die at the afflicted site which normally takes about 1-2 days (Kaplan 2011). However, the neutrophil life span can increase up to 3-5 days via blocking of apoptosis to prolong the inflammatory process. Neutrophils that do not receive the recruiting signal generally go through programmed cell death (Soehnlein 2008).

Neutrophil Function

Neutrophils primarily function in the tissues where microbial invasion or tissue damage occurs. They are highly motile and when infection or tissue damage occurs, they have the ability to migrate to the affected site and release oxidants, proteases, and other antimicrobial molecules within seconds to minutes (De Oliveira 2016). Neutrophils

express several different families of cell surface receptors for the recognition of pathogen invasion and the inflammatory environment. These include G-protein-coupled chemokine and chemoattractant receptors, Fc-receptors, adhesion receptors such as selectins/selectin ligands, integrins, and various cytokine receptors, as well as innate immune receptors such as Toll-like receptors, and C-type lectins (Futosi 2013). They are attracted to the site of the infection or inflammation by the presence of chemotactic gradients around such sites. The product of activated complement components (e.g., C3a, C5a, C657, membrane phospholipids released from damaged cells, lymphokines released from lymphocytes, products released from dendritic cells, monocytes or bacteria, platelet-derived factors) are the calling signal to neutrophils (Mathern and Heeger 2015).

Once neutrophils sense the signal of microbial invasion and inflammation, the adhesion molecules, their ligands, and vascular endothelial cells (VEC) work together to migrate through the endothelium and basement membrane through diapedesis (Langer 2009). Chemoattractant gradients guide the neutrophils through the extravascular tissue, moving by directed ameboid motion toward the infected site (Langer 2009).

Neutrophils immediately begin phagocytosis upon arrival of the infected site. The act of phagocytosis is associated with a burst of oxygen consumption and the production of hydrogen peroxide (Wittmann 2012). The ingestion of bacteria is followed by the fusion of primary and specific granules with the membrane of the phagosome and the discharge of granule content into the phagocytic vacuole (Segal 2005). While the release of the granule contents causes the elimination of foreign invaders, the overproduction of digesting enzyme can result in a systemic reaction and may also lead to multi-system organ failure (Fujishima 2016).

Neutrophil recruiting process

Human complement factor C5a is a 74-amino acid protein that is organized into four alpha helices in anti-parallel with bridging disulfide bonds (Bajic 2015). It is a fragment of complement component C5, which is released as the byproduct of complement activation (Ward 2009). C5a is a key inflammatory molecule that is generated by the three pathways of the complement cascade (Heller 1999). It is also a major recruiter of neutrophils to the sites of infections (Ward 2009). C5a peptide binds to the specific receptors on several cells such as neutrophils, macrophages, and mast cells which result in the release of histamine and other mediators. The release of histamine and other mediators recruit and allow more accessory cells to travel to the affected site (Ward 2013). The C5a peptide also upregulates the expression on C5aR1 on neutrophils (Rittirsch 2008). C5aR1 is a G-protein coupled receptor with seven transmembrane domains and three extracellular loops. C5a-C5aR1 interactions lead to changes in intracellular pH and fluid content, degranulation of neutrophils, and releasing chemotaxis factors (Rittirsch 2008). C5aR1 ligation also prolongs neutrophil survival through potentiation of the Akt/Bad pathway and decreasing activity of the pro-apoptotic caspase (Hornstein 2016). Functional responses such as reactive oxygen species (ROS) production and cytokine release are also the result of neutrophil activation through the C5aR1 ligation (Hornstein 2016).

CD177

CD177 is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that is exclusively expressed on the surface of neutrophils, neutrophilic metamyelocytes, and myelocytes (Stroncek 2004). The function of CD177 is largely unknown, but it has been

firmly established that CD177 forms a complex with neutrophil proteinase 3 and binds to platelet endothelial cell adhesion molecule 1 (PECAM-1) (Kissel 2001). This interaction does not activate inflammation process; however, it enhances neutrophil adhesion and transendothelial migration (Bayat 2010). The mechanism that mediates the migration of neutrophils through the endothelial transmigration process is currently not well established. CD177 is expressed by 30%-70% of circulating neutrophils (Matsuo 2000) and is up-regulated on surface upon stimulation, including during severe bacterial infections, and following granulocyte-colony-stimulating factor treatment (Göhring 2004). Since the CD177-positive neutrophils are the ones that travel to the infection tissue site through the transendothelium, it is a convenient target to reduce the recruitment of neutrophils (Kuckleburg 2012).

Hybrid Polymerized Liposome Nanoparticles (HPLN)

HPLN is designed to harness and enable vastly superior targeting to tumor and other specific cell surface antigens. The average diameter is about 70nm. It has a unique matrix structure that enables drug to be loaded to high concentration without leaking. It is highly flexible to display targeting peptides and proteins on its surface (NanoValent Pharmaceuticals).

GALA peptide

GALA is a 30-amino acid synthetic peptide that originates from the amino terminal segment of the H2A subunit of hemagglutinin from the influenza virus (Lear 1987) It was created to discover how the viral fusion protein sequences interact with membranes and escape the endosomal degradation (Goormagtigh 1991). The sequence includes (NH2-WEAA-LAEA-LAEA-LAEH-LAEA-LAEA-LEALAA-COOH) glutamic

acid-alanine-leucine-alanine (EALA) repeat that also contains a histidine and tryptophan residue as spectroscopic probes (Futaki 2005). The peptides are pH sensitive and have amphiphilic features. At a neutral pH, the peptides assemble themselves into random coil structures and have no effect on membrane activity. At pH 5, the peptides organize themselves into amphipathic alpha helices partitioning the amino acid side chains into a hydrophilic or a hydrophobic surface (Li 2004). This organization allows the ability to penetrate into the core of hydrophobic cell membrane lipid bilayer forming a membrane peptide pore (Nishimura 2014). During the internalization of the drug delivery via endocytosis, the GALA peptides allow avoid degradation from the endosome. In the acidic environment in the lumen of the lysosome, the peptide effectively penetrates and permeates cell lipid membranes creating membrane-spanning channels and that allow the drugs to be released in the cytosol or their destination (Mastrobattista 2002).

The goal of this proposal is to temporarily suppress the neutrophil population in the damaged tissue site by knocking down the C5aR1 expression (expressed on Neutrophils) using antisense oligonucleotides (ASO) which will be delivered by pH-sensitive GALA fusogenic hybrid polymerized liposome nanoparticles (GALA-HPLN). The first step is to incorporate the pH-sensitive fusogenic GALA peptide into HPLN. The second step is to observe the uptake and the localization of the GALA-HPLN. And finally, the third step is to examine the transient knockdown of C5aRI using a stable cell line that expresses human C5aR1 and CD177. The overarching goal of the study is to prevent unnecessary tissue damage caused by neutrophil recruitment in complications such as gout, thrombosis, lupus, stroke, myocardial infarction, and septic cardiomyopathy.

PRELIMINARY REPORT / PROGRESS REPORT

Inflammation is a complex and vital innate immune response that is activated in response to pathogen invaders, damaged cells, or tissue injury. The main purpose of inflammation is to assist indirectly in clearing out the pathogen and heal the tissues. However, the unwanted and prolonged inflammation caused by neutrophil activation can increase morbidity and mortality in diseases such as sepsis, ischemia reperfusion injury, and neurodegenerative diseases. It has been firmly established that neutrophils are the first to arrive at the tissue damage site as the inflammation initiated (Uribe-Querol 2015). Upon arrival neutrophils begin phagocytosing, releasing granules, chemokines and cytokines, amplifying the inflammation and immune response. Previous research studies demonstrated that C5a, the byproduct of complement element C5, is one of the main recruiters of neutrophils to the tissue injury site. C5a binds with C5aR1 on neutrophils, which increase upon inflammation activation, and amplify the inflammatory response.

It has been confirmed by Riley et al. (2000), Proctor et al. (2004), and Weisman et al. (1990) that the inhibition of C5a-C5aR1 axis can limit the inflammatory damage caused by neutrophil at the tissue injury site (Miettinen 2018). A study conducted by Weisman et al. (1990) showed that the size of myocardial infarction after reperfusion in rats could be reduced by inhibiting the complement system. Another study conducted by Niederbichler et al. (2006) showed that anti-C5a antibody could increase the survival rate of sepsis in mice, significantly reduce myocardial neutrophils infiltration and coronary arteriolar endothelial injury in a porcine model of cardiopulmonary by-pass and cardioplegic reperfusion. Inhibition of C5aR1 has been proven to significantly improve *in vivo* models of ischemia/reperfusion injury and sepsis.

Several drugs that target C5a-C5aR1 have been developed in recent years. However, the drugs lack cell targeting specificity such that their use produces adverse side effects. Drugs such as Eculizumab (Soliris®) were developed to treat a rare disease called paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremia syndrome (Kaplan 2014). It is a humanized antibody against C5. However, it was withdrawn from the market due to side effects that caused meningococcal infections and failure in rheumatoid arthritis trials (Mele et al. 2019; Monk 2007; Robert 2016). Another drug, called PMX-53, is a C5aR antagonist and was previously approved by FDA. It was withdrawn from the market due to nonspecific binding that cause unwanted side effects (Subramanian 2011). There are several drugs such as Avacopan (CCX-168) and DF259A that are in clinical trials as mentioned by Miettinen et al. (2018).

Currently there are no effective drugs to transiently reduce damage caused by neutrophil activation of the C5a1-C5aR1 axis (Miettinen 2018). In 2018, Miettinen and colleagues demonstrated that 67%- 82% (Figure 1) of human C5aR1 was knocked down transiently using siRNA and ASO which were delivered by CD-177, neutrophil specific, hybrid polymerized liposomal nanoparticles (HPLN). Approximately 30% of the siRNA and ASO were degraded by lysosomes after the uptake by the endocytic pathway.

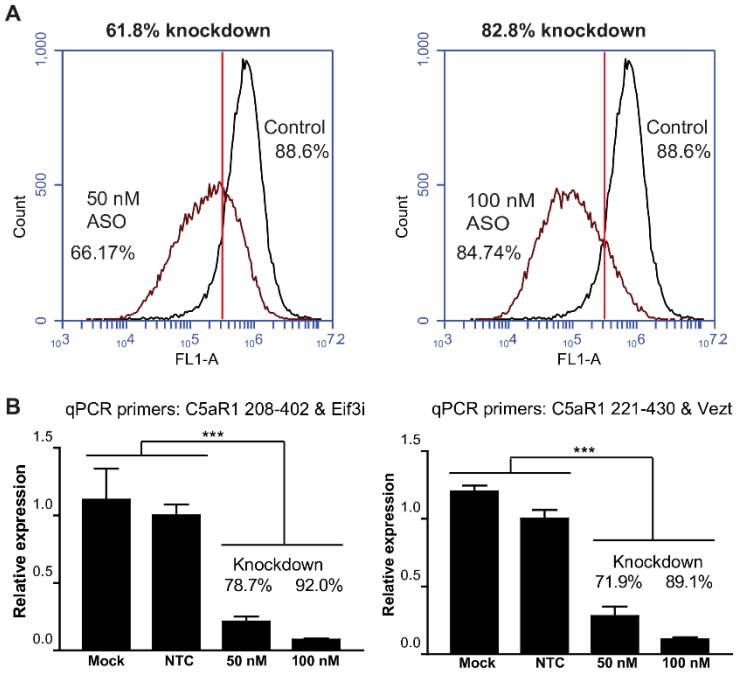


Figure 1: At approximately 72 hr post-transfection, cells were analyzed by flow cytometry to measure the relative expression of mouse C5aR1-GFP. The top (A) histogram shows 61.8 – 82.2% knockdown of mouse C5aR1 compare to the negative control. Panel B shows the reduced gene expression by qRT-PCR using two sets of mouse primers and normalized to reference genes.

Previous research done by Nishimura et al. (2014) described a pH-sensitive fusogenic GALA peptide that has the ability to escape from endosomes. In the study, GALA peptide was displayed on the surface of the Z_{her2}-BNC (bio-nanocapsule) that recognizes HER2 receptors. After incubation of GALA-His-Z_{HER2}-BNC/Liposome (LP) in HER2-positive SKBR3 cells for 48 hr, GALA-His-Z_{HER2}-BNC/LP showed (Figure 2) clear green fluorescence in the cytoplasm, indicating that calcein (green fluorescent compound that was encapsulated into the liposome as an inclusion) was successfully released from the endosomes to the cytoplasm (Nishimura 2014). Red fluorescence derived from the Lysotracker was rarely observed and this suggests that the GALA

peptide had destroyed the endosomal membranes through the process of endosomal escape; and therefore, that the endosomes had diminished (Nishimura 2014). The toxicity study also showed that each cell incubated with various particles (negatively-charged LP, His-Z_{HER2}-BNC/LP, and GALA-His-Z_{HER2}-BNC/LP) had roughly the same cell viability (greater than 90%). This demonstrated that the display of GALA peptide on the BNC (Bionanocapsule) never showed the negative effect on the cell toxicity (Nishimura 2014).

A gap in the research conducted by Miettinen et al. (2018) is that the knockdown of neutrophil-specific C5aR1 was only experimentally attempted on mouse C5aR1, but not on human C5aR1. The author claimed that ASO were too expensive to experiment with it on both human and mouse C5aR1 (Heini Miettinen-Granger, Personal Communication). Mouse-specific ASOs used by Miettinen and colleagues (2018) were specific to the murine sequence and did not correspond to or bind to the human sequences whatsoever. My grant proposal proposes to completely knock down both human and mouse CD177-specific neutrophil-C5aR1 transiently using ASO which will be delivered by pH sensitive, GALA-HPLN, to improve transiently-reduced inflammation.

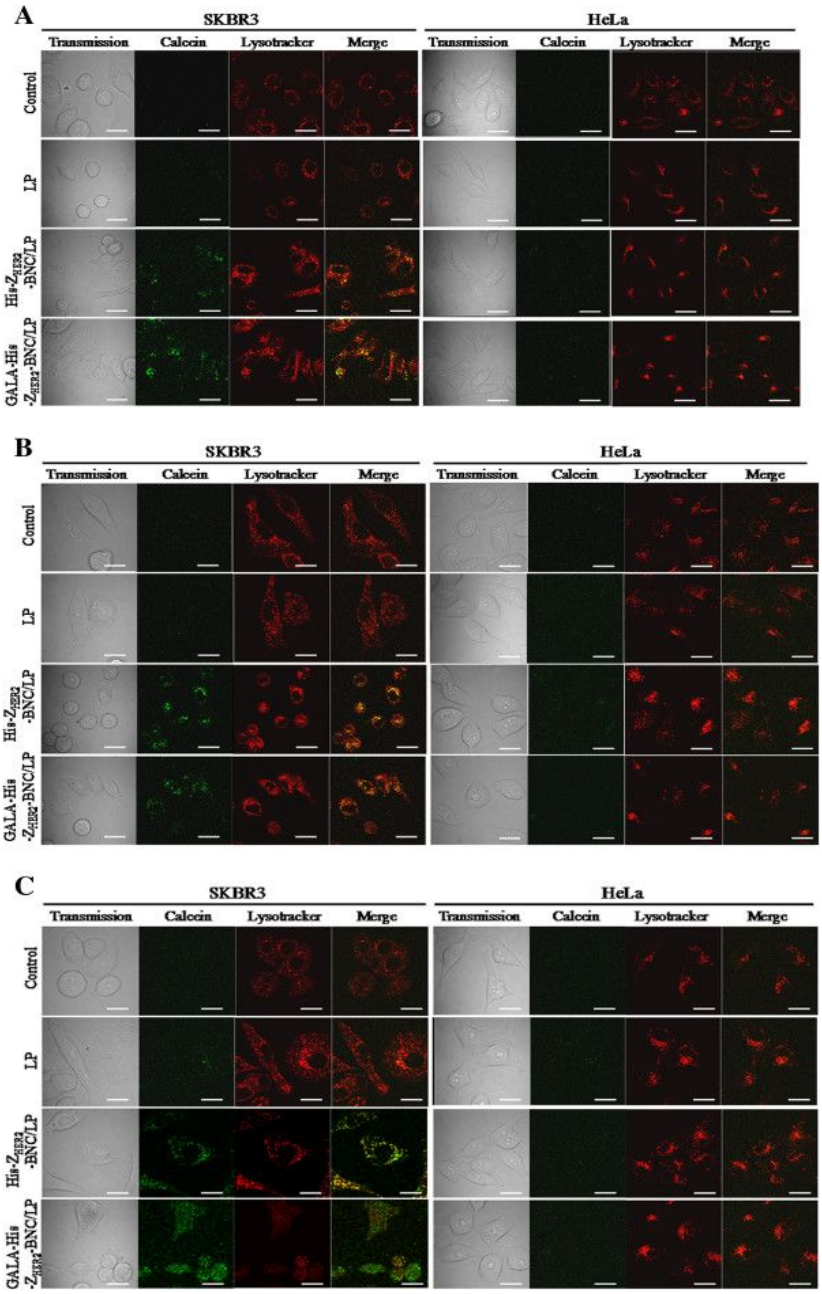


Figure 2: After incubation for 6 hr., the cellular uptake of His-Z_{HER2}-BNC/LP and GALA-His-Z_{HER2}-BNC/LP was observed in HER2-expressing SKBR3 cells. Both merged images showed yellow fluorescence, indicating that calcein was localized in endosomes. Panel C (SKBR3- 48 hr later), the GALA-His-Z_{HER2}-BNC/LP shows clear green fluorescence in the cytoplasm, indicating that the calcein was successfully released from the endosome to the cytoplasm, while His-Z_{HER2}-BNC/LP remained trapped in the endosome.

RESEACH DESIGN / METHODS

This grant proposal includes plans to modify hybrid polymerized liposomal nanoparticles by adding the GALA peptide on the surface, which will allow the nanoparticles to escape endosomes and deliver the target drug into the cytoplasm of the cell, which will result in increased knockdown of C5aR1 protein on neutrophils. To achieve this goal, ASO will be delivered by CD177 mediated-HPLN-GALA to Chinese Hamster Ovary (CHO) cells that express human or mouse CD177 and C5aR1-GFP. The knockdown of CRaR1 will be observed and compared between the degree of protein knockdown using HPLN with and without GALA. The knockdown by GALA-HPLN is hypothesized to be more efficient than the knockdown by HPLN alone.

Cell Lines

The Chinese hamster Ovary (CHO) cell line was created in 1987 in the laboratory of Dr. Ira Mellman at Yale University (Life Science Foundation 2016). Suvorova et al. (2005) previously described the construction and characterization of CHO cells that express human or mouse CD177 and C5aR1 or C5aR1-GFP. Human CD177 cDNA in the pDNA-Dual plasmid can be purchased from the DNASU plasmid repository. Mouse CD177 cDNA and pCMV6 mouse C5aR1 can be purchased from OriGene Technologies. The cloning process and plasmid information have been deposited in a public repository (Addgene 2019).

CD177 Binding Peptide

Miettinen el al. (2018) previously tested peptides that specifically bind to human and mouse CD177. They used PhD-7 and PhD-12 libraries from New England Biolabs to screen for those peptides. The table below summarizes the qualitative phage peptide

binding results obtained from their research studies. In this proposal, the highlighted human and mouse CD-177 binding peptides on Table 1 will be used for this proposed research.

Table 1: Qualitative phage peptide binding assay

Human CD177-binding peptides	CHO Hu CD177 (pfu/ml)	CHO wild-type (pfu/ml)	Fold difference
I N H Q L D T T Q I L V	1.1×10^9	5.7×10^4	1,930
F P L E T S H M S A P L	2.5×10^9	1.8×10^4	13,889
Y S S A L K T L P I F Q	2.1×10^9	5.0×10^4	4,200
K V F E Q D L L T T I L	1.7×10^9	2.6×10^4	6,538
S M Q L M T S R L T W N	5.8×10^9	7.8×10^4	7,436
N I L T T T W L P L H G	7.8×10^9	32×10^4	245
Mouse CD177-binding peptides	**CHO Ms CD177-HA (pfu/ml)**	**CHO HA-FPR (pfu/ml)**	**Fold difference**
D F Y K P M P N L R I T	1.6×10^9	1.0×10^5	15,800
W G F K P M D S L V I A	4.3×10^8	1.1×10^5	3,900

1×10^{10} phage were added to wild-type CHO cells, CHO human (Hu) CD177 cells, CHO HA-FPR cells or CHO mouse (Ms) CD177-HA cells. After 3 h incubation at room temperature, cells were washed extensively and the bound and internalized phage were eluted with 0.1 M glycine-HCl, pH 2.2, 1% Triton X-100. Titration was carried out using the manufacturer's protocol. The titers shown for each phage peptide are shown as pfu/ml. The highlighted peptides were used in the subsequent experiments.

GALA Peptide

GALA is a pore-forming, 30-amino acid synthetic peptide with a glutamic acid-alanine-leucine- alanine (EALA) repeat. It also contains histidine and tryptophan residues as spectroscopic probes. This peptide was originally designed to explore how viral fusion protein sequences interact with membranes. The peptide can be purchased from ANASPEC (Catalog # AS-62311).

Three- letters code: Trp-Glu-Ala-Ala-Leu-Ala-Glu-Ala-Leu-Ala-Glu-Ala-Leu-Ala-Glu-His- Leu-Ala-Glu-Ala-Leu-Ala-Glu-Ala-Leu-Glu-Ala-Leu-Ala-Ala
One letter code: WEAALAEALAEALAEHLAEALAEALEALAA

Figure 3: **GALA peptide sequence.**

Production of Hybrid Polymerized Liposomal Nanoparticle Particles with targeting peptides

HPLN synthesized by NanoValent Pharmaceuticals will be used as the base particles. The HPLN (Figure 3) liposomes contain hydrogenated soy L-α-phosphatidylcholine ("hydrogenated soy PC"), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ("m-Peg2000-DSPE") (all obtained from Avanti Polar Lipids, Alabaster, Alabama), N-(5'-hydroxy-3'-oxypentyl)-10-12-pentacosadiynamide ("h-Peg1-PCDA"), and N-(methoxy(polyethylene glycol)-2000)-10-12-pentacosadiynamide ("h-Peg2000-PCDA").

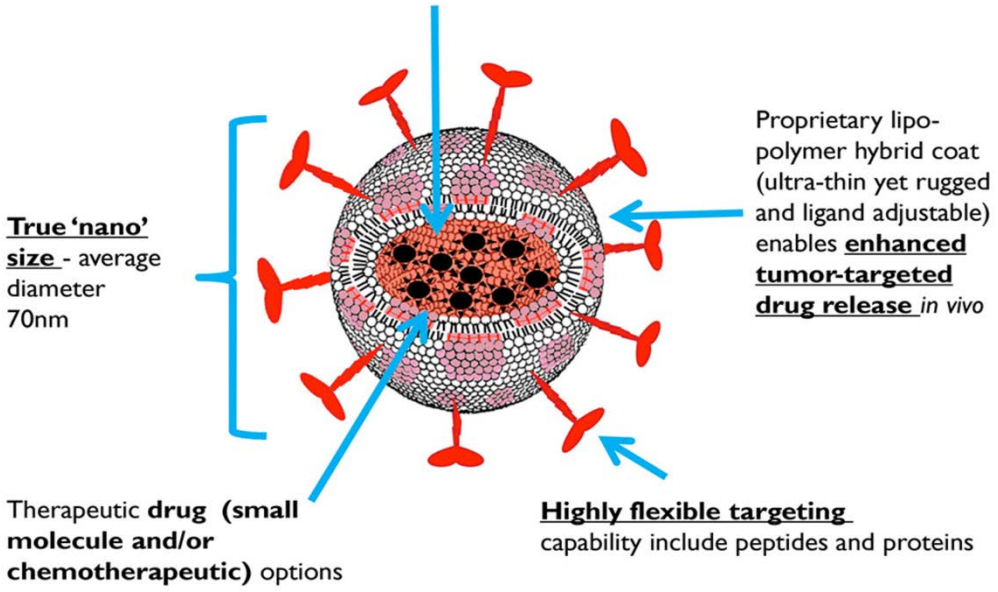


Figure 4: This figure was obtained from the NanoValent Pharmaceutical Website. This unique matrix structure allows drugs or small molecule to be deliver to the specific target without leaking and minimal toxicity.

HPLNs will be prepared by NanoValent pharmaceutical, Inc. The customized structure will have the targeting peptide (human or mouse CD177-binding peptide) and fusogenic peptide GALA on the surface with red fluorescence added. The particle size should average about 96 nm. This type of HPLN has been successfully produced and verified by Miettinen et al. (2018). The veracity of incorporation of the GALA peptide will be examined using Circular Dichroism Spectra analysis and immunofluorescence microscopy.

Table 2: Different types of HPLN that will be used in the study. GALA-scramble-HPLN and H-HPLN, GALA-M-HPLN will be used for control. GALA-H-HPLN will be used to deliver ASO to Neutrophils. The peptide sequences were obtained from Miettinen et al. (2018).

GALA-H- HPLN	Human CD177 peptide binding and GALA peptide
H-HPLN	Human CD177 Peptide Binding (Positive Control)
GALA-M-HPLN	Mouse CD177 binding peptide binding and GALA peptide
GALA-Scramble-HPLN	Scramble peptide and GALA Peptides (Negative Control)

List A. Peptide sequences

Human CD177-binding peptide:	FPLETSHMSAPLGGGC-amide
Scrambled peptide:	SLAMFLTHSPEPGGGC-amide
Mouse CD177-binding peptide:	DFYKPMPNLRITGGGC-amide

Figure 5: Peptide sequences for human, mouse, and a scrambled peptide for CD-177, adapted from Miettinen et al. (2018).

Aim 1: Production of CD177-mediated Hybrid Polymerized Liposomal Nanoparticles (HPLN) with fusogenic peptide GALA, observed using the Circular Dichroism Spectra Analysis.

Circular Dichroism Spectra Analysis

Circular dichroism (CD) spectroscopy is a spectroscopic technique where the CD of molecules is measured over a range of wavelengths. CD spectroscopy is used to study chiral molecules of all types and sizes, but it is a very important method in studying large biological molecules. A primary use is in analyzing the secondary structure or conformation of macromolecules, particularly proteins with secondary structure that is sensitive to environmental variances in temperature or pH. Circular dichroism can be used to observe how secondary structures change with environmental conditions or on interaction with other molecules. Structural, kinetic, and thermodynamic information about macromolecules can be derived from circular dichroism spectroscopy.

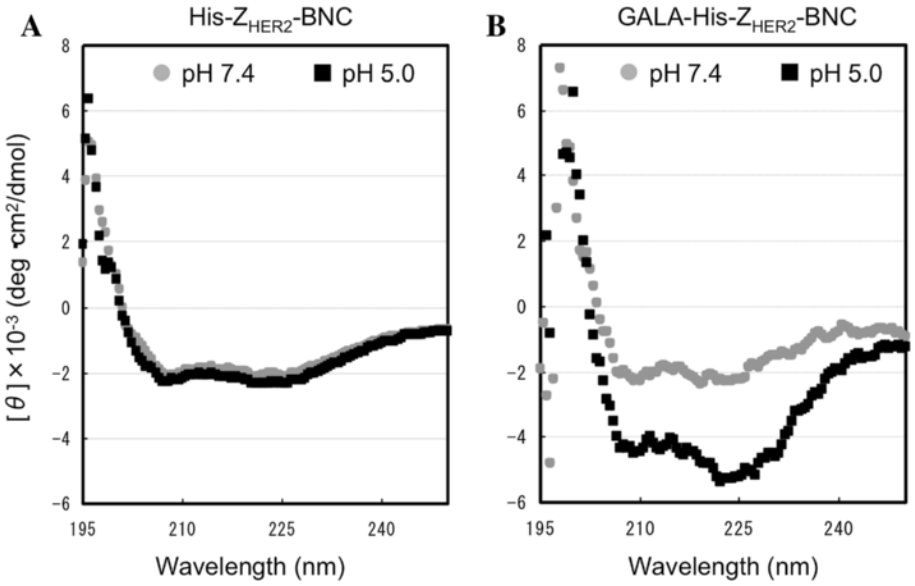


Figure 6: Circular Dichroism spectra analysis of (A) His-Z_{HER2}-BNC and (B) GALA-His-Z_{HER2}-BNC. The His-Z_{HER2}-BNC shows the same negative maxima at 208nm and 22nm are the same at pH 5.0 and pH 7.4. The GALA-His-Z_{HER2}-BNC in Panel B shows a relatively stronger negative maxima at pH 5 at 208nm and 222nm. (Figure obtained from Nishimra et al. *(*2014)

To confirm if the GALA peptide is displayed on the surface of HPLN in a functional structure, H-HPLN and GALA-H-HPLN will be measured at pH 7.4 and 5.0 over a range of wavelengths. A previous study conducted by Nishimura and colleagues (2014) used the circular dichroism spectra analysis to verify their GALA peptides displayed on the surface of bio-nanocapsule (Z_{HER2}-BNC). Employing the same techniques in this study, we will measure the H-HPLN and GALA-H-HPLN at pH 7.4 and 5.0 using J-725 K (JASCO, Japan). Detailed methods are described in Nishimura et al. (2014). Spectra will be obtained using 0.5 nm bandwidth, a scan rate of 20 nm/min and a response time of 4 sec. The CD measurement will be made using protein

concentration of 0.1 mg/ml and performed at 20 ^{o} C. The wavelength reading should range from 260 - 185 nm.

Expected Result:

At two given wavelengths, H-HPLN should have the same negative maxima (deg cm^2/dmol) at both pH 7.4 and pH 5.0. GALA-H-HPLN- should be have a relatively stronger negative maxima at pH 5.0 compare to pH7.4, which would indicate that GALA peptide on H-HPNL changes in structure from a random coil to an α-helix in response to the pH decrease. The graph seen in Figure 6 (B) of an α-helix curve should be observed. This will confirm that the GALA-H-HPLN binding peptide is pH-sensitive, forms a functional structure, and will allow for endosomal escape.

Aim 2: Examine the binding, internalization, and specificity of the HPLNs; determine if the GALA-HPLN has the ability to escape the endosome.

To observe the internalization, localization, and specificity, as well as the ability to escape the endosome, immunofluorescence microscopy, confocal laser scanning microscopy, and flow cytometry will be employed. CHO cells expressing human or mouse CD177 and human or mouse neutrophils will be used. Human whole blood will be collected in EDTA tubes from healthy volunteers with written informed consent. Mouse whole blood cells will be commercially obtained from BioIVT (www.bioivt.com). Neutrophils will be purified from the whole blood samples following the instructions of the Neutrophil Isolation Protocol from www.ncbi.nlm.nih.gov. The same antibodies that were used in recent study conducted by Miettinen et al. (2018) will be used (Table 3).

Table 3: Previous antibodies used in Miettinen et al. (2018).

Mouse monoclonal anti-human CD177	Catalog #551899 (BD BIOSciences)
Mouse monoclonal anti- HA-tag antibody	Catalog #MMS-101R (BAbCO/Covance Research Products)
Rabbit polyclonal anti-mouseCD177 antibody	Catalog#MAB8286 (R&D system)
Mouse monoclonal anti-human LAMP 2 antibody	Catalog sc-18822 (Santa Cruz, INC.)
Mouse monoclonal anti-hamster LAMP2 antibody	Donation from Dr. Bruce L. Granger, Ulthayakumar 1995
Secondary Alexa Fluor 488 goat anti-rabbit antibody	Catalog # A-11008 (Molecular Probes)
Human CD177 peptide binding	GenScript (Peptide sequence shown in Figure 4)
Scrambled Control peptide binding	GenScript (Peptide sequence shown in Figure 4)
Mouse CD-177 peptide binding	GenScrip t(Peptide sequence shown in Figure 4)

Aim 2a: Human-CD177 peptide binding on GALA-H-HPLN binds specifically to human-CD177 positive CHO cells.

This experimental observation will be conducted using immunofluorescence microscopy. CHO cells expressing human CD177 will be grown on glass coverslips. The cells will be incubated for 3 hr at 37 ^{o}C with 100 ug/ml GALA-H-HPLN and GALA-scramble H-HPLN. GALA-scramble H-HPLN and CHO cells that do not express human CD177 will be used as a negative control. Cells will be washed to remove the unbound HPLNs, fixed with paraformaldehyde, permeabilized with saponin and gelatin. CD177 will be detected using a mouse anti-human CD177 antibody (primary antibody) and an Alexa Flour 488 goat anti-mouse as the secondary antibody (Figure 7). H-HPLN are fluorescent red. The fluorescence intensity comparison between the expression level of CD177 and GALA-H-HPLN will be observed under Florescence microscopy.

To further verify the that GALA-H-HPLN is binding specifically to CD177, and not randomly to other receptors, we will include a blocking peptide control. Before

proceeding with the staining protocol, the antibody (CD177-peptide binding) will be neutralized (incubated with an excess of peptide CD177). The antibody that is bound to the blocking peptide will no longer available to bind to the epitope present on the cell. The neutralized antibody can then be used side-by-side with the antibody alone, and the results are compared. By comparing the staining from the antibody-blocked versus the antibody alone, we would expect to see that staining is specific. To clarify, in the samples treated with blocking peptide, antibody staining will be absent (blocked).

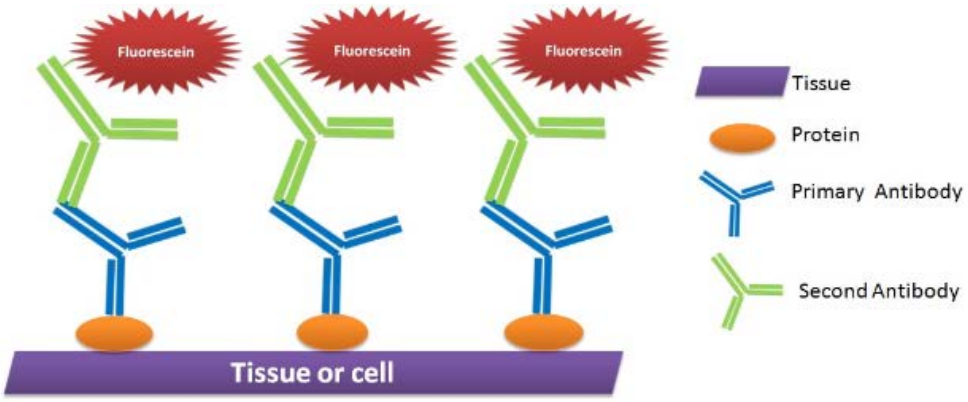


Figure 7: Tissue or cell→CHO cells, Protein→CD177, Primary antibody→ mouse anti-human CD177 antibody, Secondary Antibody →Alexa Flour 488 anti-mouse antibody (Figure retrieved from www.sinobiological.com).

Expected result:

There should be a direct correlation between the florescence intensity between the CD177 and GALA-H-HPLN (Figure 8). Where GALA-HPLN is binding to CD177, we would expect to see the red and green fluorescence merged, producing yellow, which will

be an interaction of very close proximity. I will use the parental CHO cells (not transfected with CD177) as a base line and normalize CD177-expressing CHO cells against the parental cell line to demonstrate that the fluorescence observed coming from the CD177 CHO cells is the result of the stable transfection. The GALA-scramble H-HPLN should not be seen at all in the cells. This experiment will verify that the CHO cells express CD177 peptide and that GALA-H-HPLN has the ability to specifically bind on CD177.

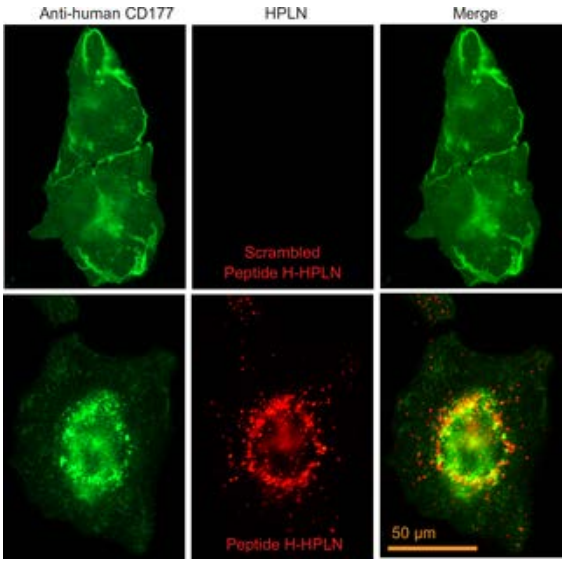


Figure 8: HPLNs displaying Human CD177 Peptide binding binds human CD177 on cells (Miettinen 2018). The figure depicts the type of result that is expected. The distribution of CD177 and peptide H-HPLN is proportional, whereas the scrambled peptide H-HPLN is not present in the cells.

Aim 2b: Internalization of GALA-M-HPLN by CHO cells that express Mouse CD177-HA will be confirmed using subtilisin.

Subtilisins are proteolytic enzymes that are defined by their catalytic mechanisms. To confirm the GALA-M-HPNL are successfully internalized, CHO cells will be

incubated with and without subtilisin. CHO cells expressing mouse CD177-HA will be incubated with 100 ug/ml of GALA-M-HPLN for 2 hr on ice to allow the binding. The cells will be washed to remove the unbound HPLN. The cells will be further incubated for 1 hr at 37 ^{o}C or on ice to allow for internalization. Incubation on ice will inhibit the internalization. Cells will be treated with subtilisin to remove surface-bound HPLN particles or treated with buffer alone. Cells will then be fixed, permeabilized, and stained with primary and secondary antibodies as described above. Mouse CD177-HA will be stained with a mouse anti-HA antibody and Alexa Fluor 488 goat anti-mouse secondary antibody. Cells will be observed under immunofluorescence microscopy.

Expected result:

Cells that are incubated on ice with or without subtilisin should display an absence of HPLN inside in the cells. The low temperature does not allow HPLN to be internalized. The surface-bound HPLN should be cleaved off by subtilisin; this will facilitate the visualization of internal fluorescence and distinguish from fluorescence originating on the cell surface. This is a negative control. Cells that are incubated on 37 ^{o}C with or without subtilisin should have the same amount of the HPLN particles inside the cells. This will demonstrate that the HPLN particles are internalized successfully and are protected from the protease activity.

Table 4: The table represents the expected results from this experiment using subtilisin enzymatic cleavage.

Temperature (1hr incubation)	Anti- Human CD177 (Green)	GALA-H-HPLN (Red)
4 ° C	Green	None seen- not internalized
4 ° C (subtilisin)	None seen (negative control)	None seen
37 ° C	Green (positive control)	Red – HPLN inside the cells
37 ° C (post –subtilisin)	None	Red- HPLN inside the cells
37 ° C (subtilisin preincubation)	None (negative control)	None -Cleaved by subtilisin

Aim 2c: Observe GALA-HPLN specificity for neutrophils isolated from human and mouse whole blood.

Human whole blood samples will be collected in EDTA from healthy volunteers with written informed consent. Mouse whole blood samples will be purchased from BioIVT (www.bioivt.com). The neutrophils isolated from the whole blood samples will then be incubated with GALA-H-HPLN or GALA-M-HPLN at 100 ug/ml concentration for 1 hr at 37 ºC. Neutrophils will be resuspended in RPMI-1640 supplemented with 10% fetal bovine serum. Cells will be adhered onto a glass slide using a Cytospin centrifuge. Human cells will be fixed in methanol and stained with primary antibody (anti-human CD177) and secondary antibodies (Alexa Flour 488 goat anti-mouse secondary antibody). Mouse cells will be fixed in methanol and stained with rabbit anti-mouse CD177 polyclonal antibody and Alexa Fluor 488 goat anti-rabbit secondary antibody. HPLN will fluoresce red. The slides will be observed using

immunofluorescence microscopy. GALA-M-HPLN (mouse CD177 peptide biding) incubated with human neutrophils or GALA-H-HPLN with mouse neutrophils will be used as negative controls.

Expected result:

The red fluorescent protein conjugated to the HPLNs should be observed inside human or mouse neutrophils. The negative control should not have any red fluorophore inside the neutrophils. This will confirm that the HPNLs are species-specific, able to bind to specific target receptors, and are internalized.

Aim 2d: Flow cytometry to corroborate confirmation of the GALA-H-HPLN specificity to human CD177-positive-neutrophils

Flow cytometry is a widely used method to analyze the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population. Cells pass through a laser beam one cell at a time. Cells are often labeled with fluorescent markers so that light is first absorbed and then emitted in a band of wavelengths. Light scattered from the cells is detected by forward scatter and side scatter. Fluorescence detectors measure the florescence emitted from positively stained cells. All light is collected by the detector and processed through the electronics component of the flow cytometer.

To verify that only CD177 positive neutrophils are positive for H-HPLN, human white blood cells will be incubated at 37^{o} C with GALA-H-HPLN to allow uptake. To determine that positive CD177 cells take up the HPLN, I will stain all the cells with

primary antibody, anti-human CD177 antibody, and secondary antibody, Alexa Fluor 488 goat anti-mouse antibody. The samples will be measured with flow cytometry.

Expected result:

We should observe GALA-H-HPLN only in CD177 positive neutrophils (Figure 9) and not on other leukocytes such as monocytes or lymphocytes. This will verify that GALA-H-HPLN is neutrophil specific.

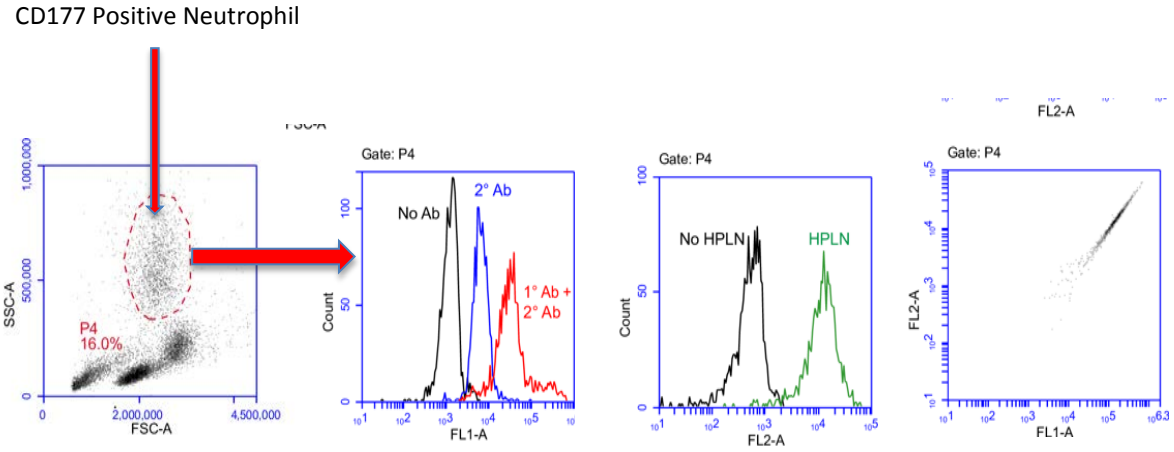


Figure 9: The second histogram shows distinct peak of CD177 (Red) positive neutrophil population. The third histogram shows distinct peak of HPLN (Green). The fourth histogram shows the double positive on the top right corner to verify that only CD177 positive neutrophils are positive for HPLN. Figure reproduced from Miettinen et al. (2018).

Aim 2e: GALA-H-HPLN Endosomal Escape

CHO cells expressing human CD177 or HA-tagged mouse CD177 (HA tag, YPYDVPDYA) will be used to assess endosomal escape in this experiment. A complex consisting of conjugated GALA-HPLN or HPLN with anionic with LP (COATSOME EL

-010A), that has previously been shown to lack the ability to escape the endosome (Jung 2008) will be developed following previously reported research methods. To visualize the destination of the particle inclusions, a green fluorescent compound, calcein, will be encapsulated into the LP. Six types of particles; GALA-H-HPLN/LP, H-HPLN/LP, H-HHPLN, GAL-H-HPLN, LP (control), and mock transfected CHO cells will be incubated in CHO cells that express human or mouse CD177 (Table 5). The control will consist of CHO cells with no HPLNs. The endosome will be stained with blue fluorescent lysotracker, blue DND 22 (Invitrogen Life Technologies, Carlsbad, CA, USA). A yellow color should be observed when the blue florescent lysotracker and the green fluorescent from calcein are merged (Jung 2008). This will be a sign of endosome-colocalized particles containing calcein. The cellular kinetics will be observed using a confocal laser scanning microscope. The observation will be made at various times, including at 0 hr, 3 hr, 6 hr, 24 hr, and 48 hr of incubation. The appropriate culture conditions, including temperature, culture medium, CO_2, have previously been described by Nishimura et al. (2014).

Table 5: Six particles will be incubated in CHO-H-CD177/C5aR1.

Incubate at 37 º C		**Observation Time**
GALA-H-HPLN/LP	CHO-H-CD177/C5aR1	0 hr , 3 hr, 6 hr, 24 hr, 48 hr
H-HPLN/LP	CHO-H-CD177/ C5aR1	
H-HPLN (Pos control)	CHO-H-CD177/ C5aR1	
GALA-H-HPLN	CHO-H-CD177/ C5aR1	
LP (Neg control)	CHO-H-CD177/ C5aR1	
None (Neg control)	CHO-H-CD177	

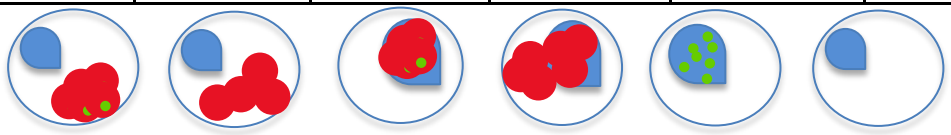
Expected Result:

We should observe the uptake of HPLNs by CHO cells in the first few hours. As the incubation period increases, we should observe colocalization of HPLN and endosomes. Eventually we should observe the GALA-H-HPLN/LP and GALA-H-HPLN in the cytoplasm successfully released from the endosome.

Table 6: Aim 2e expected result. GALA-H-HPL and GALA-H-HPLN/LP should successfully escape endosome.

HPLN-Red, Endosome-Blue, LP-Green, Two Colors - Yellow, Three Colors- White

	GALA-H-HPLN/LP	GALA-H-HPLN	H-HPLN/LP	H-HPLN (Pos)	LP (Neg)	No HPLN (Neg)
Endosome	Blue (Endo, no colocalization)	Blue (Endo, no colocalization)	White (HPLN/LP, Endo colocalization)	Yellow (HPLN,Endo, colocalization)	Yellow (LP and Endo colocalization)	Blue (Endo)



Aim 3: Examine the knockdown of human C5aR1 using ASO

The lifespan of a neutrophil *in vitro* is about 8 hr (Pillay 2010) which would be too short to observe the knockdown of C5aR1. The knockdown delivery by ASO is expected to occur within 4-72 hr after administration (Miettinen 2018). For this reason, the knockdown of C5aR1 will be examined using a stable cell line. CHO cells that express various combinations of human or mouse C5aR1-GFP and CD177 will be used. The protein knockdown will be measured by flow cytometry and mRNA knockdown will be measured by qRT-PCR.

Mouse C5aR1-ASO1, previously confirmed by Miettinen and colleagues (2018), will be used to knockdown mouse C5aR1. Human C5aR1-ASOs were designed against the human RefSeq C5aR1 gene (NM_001736.3). The gene is comprised of only two (2) exons and the genomic sequence is 2342 nt in length. It was necessary to design new ASOs against the human sequence because the mouse-specific ASOs that were described

and previously tested by Miettinen et al. (2018) were species-specific to the murine sequence and did not correspond to or bind to the human sequences whatsoever (Heini Miettinen-Granger, Personal Communication). The phosphorothioate modification was chosen to both mirror the previous work by Miettinen et al. (2018) and for the positive properties that such modification would confer to the ASOs; namely, resistance to nuclease digestion and activation of RNase H pathways (IDT, 2011).

We will transfect CHO cells that express variable combination receptors with no ASO (no DNA, mock transfected), Non-targetable ASO (100 nm), 100 nm of ASO (Miettinen 2018), and 100 nm of ASO with GALA-HPLN according to the table below.

Table 7: NTC- non-targeting control. CHO cells that express mouse or human CaR1-GFP/CD177 will be transfected with 100 nm ASO or 100 nm ASO GALA-HPLN. Non-targetable ASO and no ASO will be use as control.

C5aR1-GPF/CD177	100 nm ASO	100 nm ASO (GALA)	100 nm NTC	No ASO
Mouse	Pos Control	Experiment Group	Neg Control	Neg Control
Human	Pos Control	Experiment Group	Neg Control	Neg Control

The transfection process will be carried out using Trans IT®-Oligo transfection reagent (Mirus Bio, LLC) following the manufacturer's protocol. Culture medium will be replaced every 24 hr. Once the incubation period reaches 72 hr, cells will be removed from the culture plate using trypsin-EDTA. Cells will be centrifuged and resuspended in PBS containing 10% of fetal bovine serum and 1 ug/ml propidium iodide (detecting dead cells). Cell suspensions will be analyzed using flow cytometry for the expression of

C5aR1-GFP. We will exclude clumped cells, highly vacuolar cells, and dead cells. The wavelengths of the exciting laser, detecting laser for GFP (488 nm excitation laser and 533/30 nm FL1 filter), and detecting laser for propidium iodide fluorescence (585/40 nm FL2 filter) are previously described by Miettinen et al. (2018).

Table 8: Mouse C5aR1 LNA™ GapmeR ASOs will be obtained from Exiqon; these ASOs were validated by Miettinen et al. (2018). Human C5aR1 ASOs were designed against the human RefSeq C5aR1 gene (NM_001736.3). The phosphorothioate modification was chosen and the position of phosphorothioated DNA bases are indicated (*). Mouse ASO1 and Human ASO1 will be used to carry out the experiment.

C5aR1 ASO	Sequence	order number
Mouse ASO1	5'-A*A*C*G*G*T*C*G*G*C*A*C*T*A*A*T-3'	580726-1 (nt 594-579 of NM_001173550.1)
Mouse ASO2	5'-A*A*C*G*G*T*C*G*T*G*A*A*C*A*G*G-3'	580726-2 (nt 479-464 of NM_001173550.1)
Human ASO1	5' A*A*C*A*C*C*C*A*G*C*A*G*A*A*A*A*G*C*C*G*G*T*C 3'	spanning nt 465-446 of NM_001736.3
Human ASO2	5' A*C*A*G*G*A*A*A*G*G*A*A*G*G*G*G*T*A*A*T*G*G*G*T*C 3'	spanning nt 569-549 of NM_001736.3

To further explore the effects of the experiment, mRNA levels of C5aR1 at 72 hr post-transfection with ASO, and reference genes, Eif3i and Vezt, will be evaluated using a quantitative RT-PCR method. These gene are ideal to use as reference genes for this experiment because mRNA level of Eif3i is higher while Vetz is lower compare to C5aR1 mRNA levels in mouse (Miettinen 2018). The primers to amplify the murine genes have been validated in previous research (Bruehl 2001). The reference genes will be measured to confirm that the internalization of the HPLN does not interfere with the activity of neutrophils, does not produce toxicity, and for normalization.

Total RNA will be purified from 72 hr post-transfection from the same cells that will be used for flow cytometry. The Qiagen RNeasy Plus micro kit (catalog # 74034) will be used, following the manufacturer's protocol to obtain total RNA. Total RNA will be transcribed into cDNA using the Qiagen miScript II RT kit (catalog # 218160). Quantitative PCR will be run using 2x QuantiTect SYBR green mix (Qiagen; catalog # 204143), mouse or human primers, and RNA transcribed cDNA. Primers for CHO housekeeping genes will be used from the study done by Bahr and coworkers (Bahr 2009). Two different sets of murine C5aR1 primers will be used to control for possible annealing and experimental variability (shown in Table 7). The primers were validated by previous research (Miettinen 2018). Likewise, two different sets of human C5aR1 will be used. These primers are shown in Table 8 below and the cycling parameters are displayed in Table 9. The data will be analyzed using the ΔΔCq calculation using the technical notes provided by Dharmacon and based on the, MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Guidelines (Bustin 2009).

Table 9: qRT-PCR primer sequences for Mouse C5aR1 and housekeeping genes used by Miettinen and colleagues (2018). These primers sequences will be used for current proposal.

qRT-PCR primer sequences	Quantitative PCR cycling

Mouse C5aR1 nucleotides 208-402 (195 bp)
Forward primer: 5'-GTCACCGCCATCTGGTTTCT-3'
Reverse primer: 5'-ACGGTCGGCACTAATGGTAG-3'

Mouse C5aR1 nucleotides 221-430 (210 bp)
Forward primer: 5'-GGTTTCTGAATCTGGCGGTG-3'
Reverse primer: 5'-ACCAGATGGGCTTGAACACC-3'

Control genes:

CHO Eif3i (166 bp)
Forward primer: 5'-CCACAACTTCCACCAGGATT-3'
Reverse primer: 5'-ATGCGGACGTAACCATCTTC-3'

CHO Vezt (200 bp)
Forward primer: 5'-GTGTGAAAGTGGGGCTGAAT-3'
Reverse primer: 5'-GTTCCTGCATGGTGGTGAAT-3'

Conditions	Number of cycles	Ramp	Duration	Target
Preincubation	1	4.0ºC/s	900 s	95ºC
Amplification	45	2.2ºC/s	15 s	94ºC
		2.2ºC/s	15 s	55ºC
		4.0ºC/s	15 s	70ºC
Melting	1	4.4ºC/s	10 s	95ºC
		2.2ºC/s	60 s	50ºC
		-	-	95ºC
Acquisition Mode: Continuous, 5 readings/ºC				
Cooling	1	2.2ºC	60s	37ºC

Table 10: Human C5aR1 Primers were designed against the human RefSeq C5aR1 gene (NM_001736.3) using Primer-Blast (NCBI 2019).

Human C5aR1 RT-PCR Assay
Primer pair: 134 bp
Hu C5aR1 forward: 5'-AGCCCAGGAGACCAGAACAT-3'
Hu C5aR1 reverse: 5'-AGGATGTCTGGAACACGCAG-3'

Table 11: Quantitative qRT-PCR Cycling Parameters for Human C5aR1

Conditions	Number of cycles	Ramp	Duration	Target (°C)
Hot Start	1	4 °C/s	15 min	95 °C
Denature		2 °C/s	15 sec	95 °C
Anneal		2 °C/s	30 sec	60 °C
Elongate*	40	4 °C/s	15 sec	72 °C
Melt Curve		4 °C/s	10 sec	95 °C
	1	2 °C/s	60 sec	50 °C
Cool	1	2 °C/s	60 sec	37 °C

* Single acquisition at the end of the cycle

Expected Result:

In the flow cytometry portion of the experiment, we should expect to observe an approximately 95-99% decrease in the fluorescence of human and mouse C5aR1-GFP levels compared to the negative controls. We will measure the total area under the curve in the GFP-negative gate and compare it to the area under the curve of the GFP-positive gate (Negative Control- no ASO). In the qRT-PCR result, we expect to observe 95-99% knockdown of human and mouse C5a receptor mRNA compare to the negative control. The expression of reference genes should not be affected by ASOs transfections and should remain stable over the course of the experiment.

Conclusion and Future Research

Neutrophils perform a critical role in the immediate, primary immune response. In contrast to their positive critical role in responding to immune challenge, they can also do great damage to the effaced tissue sites, cause chronic illness, or even death in certain instances. The ability to control inflammation is critical in certain conditions such as sepsis or myocardial infraction. Previous research by Miettinen et al. (2018) demonstrated that it is possible to develop a drug that will transiently reduce neutrophil-related inflammation. Miettinen et al. (2018) were able to reduce protein levels of mouse C5aR1 by up to 84% using neutrophil-specific (CD177) HPLN that was used to deliver ASO targeting murine C5aR1. In this proposal, I will incorporate a GALA peptide, which will help the HPLN escape the endosome, onto the surface of HPLN in the hope of completely and transiently knocking down both human and mouse C5aR1. The ability to transiently, completely, stop neutrophil activity during inflammation could be therapeutic in deadly illnesses such as ischemia reperfusion injury or neurodegenerative disease. In future research, this can be tested in mice or even in human neutrophils if a protocol can be developed to sustain the human neutrophil life span *in vitro*. This technique can be further modified to deliver other drugs. The capability to specifically and successfully deliver a drug to the target site with minimal side effects could improve treatment of diseases. Overall, the goal of all scientific research is to “enhance health, lengthen life, and reduce illness and disability” as NIH states on its mission statement.

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