Quantifying Phagocytosis – studies on the antibody response during invasive streptococcal infections

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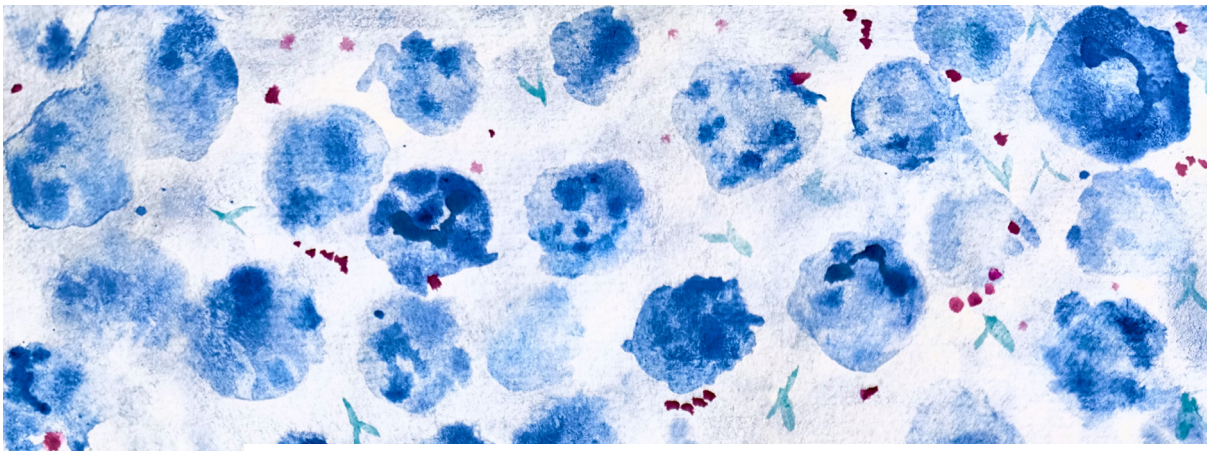

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– studies on the antibody response during invasive streptococcal infections

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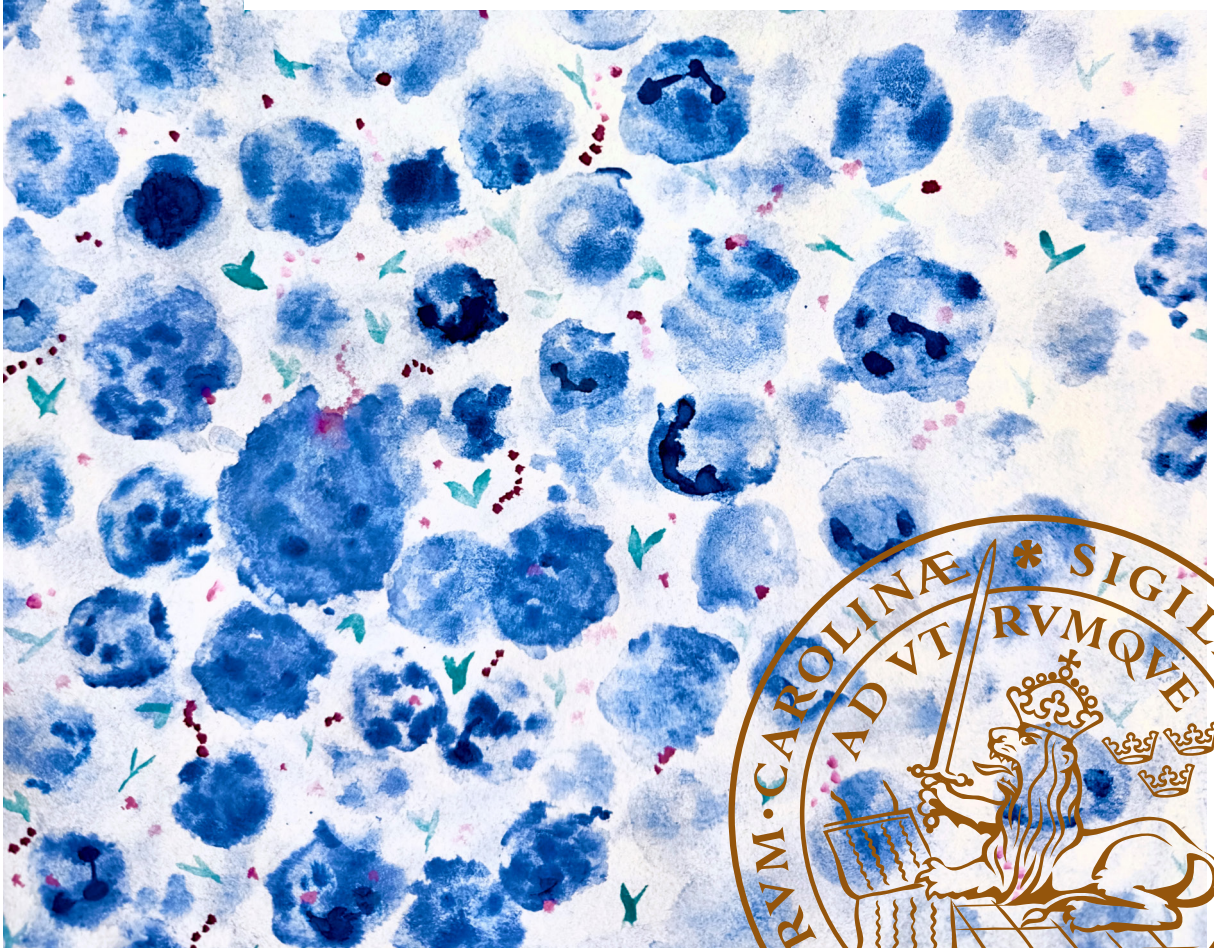

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (Ph.D.) at the Faculty of Medicine at Lund University to be publicly defended on 1st of March 2024 at 09.00 in Belfragesalen, Biomedical Center (BMC), Lund, Sweden.

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Title and subtitle: Quantifying Phagocytosis – studies on the antibody response during invasive streptococcal infections

Abstract: The interaction between our immune system and pathogens encompasses a complex spectrum from symbiosis to potentially life-threatening diseases. Throughout evolution, our immune system has evolved numerous strategies such as phagocytosis to combat infections and protect us from diseases. Phagocytosis involves the engulfment of pathogens by our immune cells and can be facilitated through opsonization, a phenomenon where antibodies bind to the prey. However, pathogens like *Streptococcus pyogenes* and *Streptococcus dysgalactiae* have developed countermeasures against it.

These pathogens cause significant global health burdens, yet vaccines are unavailable, and the development of natural immunity is not fully understood. Opsonic antibodies are considered crucial in immunity, but quantifying phagocytosis remains challenging due to a lack of standardized, reproducible methods across laboratories and systems. Addressing this gap is vital not only for enhancing the quantification of opsonic antibody response in streptococcal infection but also for benefiting various research fields where phagocytosis is a pivotal and widely measured functional outcome. This thesis aims to develop a universal and robust method for quantifying phagocytosis and assessing antibody function in streptococcal infections.

A novel method for quantifying phagocytosis is established. It is based on the principles of the Hill equation and collision theory (Paper I). This versatile, quantifiable, and robust approach enhances phagocytosis assessment and provides insights into opsonic capacity of antibodies. Additionally, a biophysical model is developed that predicts the binding of antibodies against the streptococcal M protein (Paper II). Together, the opsonic capacity of antibody responses in patients with invasive infections of *S. dysgalactiae* and *S. pyogenes* is subsequently evaluated (Paper III and IV). Although limited study populations, instances of non-opsonic antibodies are discovered for both pathogens, which can potentially be linked to recurrent invasive *S. dysgalactiae* infections. The results indicate that higher antibody titers do not always equate to opsonic responses, calling for a nuanced understanding of antibody function in streptococcal infections. Furthermore, patients with *S. pyogenes* infection unexpectedly developed opsonic antibodies effective against other strains as well. These findings contribute to the current understanding of natural immunity by suggesting not only type-specific antibodies may convey *S. pyogenes* immunity.

This thesis provides insights into the development of immunity against invasive *S. pyogenes* and *S. dysgalactiae* infections and introduces novel methods for enhancing the assessment of phagocytosis and antibody function. In conclusion, it advances our understanding of how to quantify antibody-mediated phagocytosis and its importance in the context of invasive streptococcal infections.

Key words: Phagocytosis, Hill equation, *Streptococcus pyogenes*, *Streptococcus dysgalactiae*, antibodies, invasive infection

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Dedicated to my family

Remember, be kind and stay curious,
then great things will happen.

Preface

Thank you!

Thank you for holding this thesis in your hands, I am honored. Regardless of whether you are reading this as a friend, colleague, family member, or fellow phagocytosis enthusiast like myself, I am grateful for you taking the time to explore my doctorate thesis. You do not need to read it cover to cover, but by engaging in this thesis I know you care.

In fact, this thesis is all about caring; caring for research and proper science, caring for phagocytosis and accurate quantification, and last but not least, caring for people suffering from invasive infections. I hope you will find it interesting and inspiring. Hopefully, some of you will not completely agree with me and provide additional perspectives. I warmly welcome any discussions that might advance science further, and with any luck, lead to intriguing collaborations in the future.

I particularly encourage you to read the acknowledgments section at the end. To me, it is the most important part of my thesis, where I express my sincere gratitude to those who have been fundamental in this journey.

This thesis means a lot to me, and your interest in it is deeply appreciated.

So, once again, thank you!

Therese de Neergaard,

The author

Lomma, 18th of January, 2024

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Abstract

The interaction between our immune system and pathogens encompasses a complex spectrum from symbiosis to potentially life-threatening diseases. Throughout evolution, our immune system has evolved numerous strategies such as phagocytosis to combat infections and protect us from diseases. Phagocytosis involves the engulfment of pathogens by our immune cells and can be facilitated through opsonization, a phenomenon where antibodies bind to the prey. However, pathogens like *Streptococcus pyogenes* and *Streptococcus dysgalactiae* have developed countermeasures against it.

These pathogens cause significant global health burdens, yet vaccines are unavailable, and the development of natural immunity is not fully understood. Opsonic antibodies are considered crucial in immunity, but quantifying phagocytosis remains challenging due to a lack of standardized, reproducible methods across laboratories and systems. Addressing this gap is vital not only for enhancing the quantification of opsonic antibody response in streptococcal infection but also for benefiting various research fields where phagocytosis is a pivotal and widely measured functional outcome. This thesis aims to develop a universal and robust method for quantifying phagocytosis and assessing antibody function in streptococcal infections.

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Layman's Summary

You are composed of, among other things, millions of heroes who together protect you from a multitude of diseases and dangerous infections every day. Collectively, they form your immune system, without it you would not survive for long. The immune system can be compared to a very powerful and highly skilled security team. They exist in your body to protect you against harmful invaders such as bacteria and viruses, but also to detect cancer and clear out aged, non-functioning cells that are to be replaced. Their team consists of many different individuals with various approaches to keeping our body in balance and destroying invaders. One such mechanism is to eat up the invaders, thereby killing them. This process is called phagocytosis and is vital in the fight against infections. To facilitate the detection of these invaders, there are special tags that mark them for destruction, known as antibodies. These antibodies can be developed after an infection or vaccination, making it easier for the immune system to find and destroy the bacteria or virus so we do not get sick.

However, some really tricky bacteria have developed ways to avoid this defense mechanism, of which *Streptococcus pyogenes* is one of the most dangerous. It is known for causing tonsillitis, scarlet fever, and superficial skin infections such as erysipelas, but historically has also been a major contributor to mortality in puerperal fever and pneumonia. Even today, it affects over 700 million people annually and kills more than half a million each year. It can cause life-threatening infections where it invades deep into our tissues and into our bloodstream, known as invasive infections. Fortunately, invasive infections are not as common as mild ones, but together they pose a significant global problem, earning the alias "killer bacteria" and "flesh-eating bacteria". It can cause sepsis, deep skin infections such as necrotizing fasciitis, and other serious conditions such as STSS (streptococcal toxic shock syndrome). *Streptococcus dysgalactiae*, a relative of *S. pyogenes*, can also cause these infections but has historically been less common. Unfortunately, it is becoming increasingly common, but unlike *S. pyogenes*, which can infect anyone including young healthy individuals, *S. dysgalactiae* more often infects older individuals with multiple underlying diseases. Its invasive infections can also recur, which virtually never happens with a healed invasive *S. pyogenes* infection.

Currently, there is no vaccine to protect us against these terrible diseases, so what healthcare can do is support the body if it becomes ill and try to kill the bacteria with antibiotics. In some very serious cases, infected body parts may even need to be amputated to prevent the infection from spreading. Antibodies from healthy individuals can also be given to help the immune system find the bacteria and the toxins they secrete. In order to develop vaccines and more drugs against these different infections in the future, we first need to better understand how our immune system interacts with the bacteria. Why can some people carry the bacteria in their throat or on their skin without even getting sick while others die from it? Why do

we get long-term protection after an invasive infection but superficial infections can recur several times? Much research suggests that antibodies, which contribute to bacterial killing via phagocytosis, play a significant role but much is still unknown, especially regarding invasive infections.

The purpose of this doctoral thesis is to contribute with tools for studying and measuring these interactions to increase the understanding of our immune system's fight against these infections. In fact, it is difficult to measure phagocytosis and antibody function in a reliable way that can be compared between different research groups, over time, and between different prey. This thesis consists of four individual projects that together address this. In the first project of the thesis a method for measuring phagocytosis is developed that is based on a mathematical model (Hill's equation) used in other areas, such as evaluating the effectiveness of a drug. The method is easy to use and can improve many already available methods with only minor changes. The second project is a model based on advanced mathematics to predict how antibodies bind to a very important surface protein (M protein) in *S. pyogenes.* It can be used to calculate how an additional treatment with a specific antibody mixture would likely contribute to the immune system's ability to mark *S. pyogenes* in different individuals with different antibody profiles. In the third and fourth projects, these two methods are used to study whether antibodies develop in patients affected by invasive *S. dysgalactiae* and *S. pyogenes* infections. The studies are small with few patients but unique thanks to the level of detail and accuracy with which we measure the ability of the antibodies in the patients' blood to improve the phagocytosis of these bacteria.

In some patients, we found that more antibodies did not always mean better phagocytosis of these bacteria. This is important because it suggests that it is not just about the number of antibodies, but also how well they work. In other words, quality over quantity. This could be a reason why *S. dysgalactiae* can cause recurrent invasive infections. In patients with *S. pyogenes* infections, some individuals developed antibodies that were effective against several strains of *S. pyogenes*. This was an unexpected and exciting discovery that can contribute to increasing the understanding of how our immune system works to protect us long-term against more than one specific strain of *S. pyogenes*. Something that has long been thought impossible. Together, our findings show how important it is not only to study the presence of our immune system in various diseases but especially its function. What would you choose yourself? A security system that might be large but you do not know if it works, or a security system that you not only know it works but also how well it works against different intruders? In the future, I hope it will be possible to measure the function of one's immune system to tailor which vaccines and drugs one needs and when they are needed. My hope is that this doctoral thesis will be able to contribute to parts of the foundation that such a journey would need to begin with. A journey that hopefully would be the start of something much bigger and even better than what we can imagine with today's knowledge.

In conclusion, this doctoral thesis gives us new tools and insights into how our bodies fight certain bacterial infections. Understanding this could lead to better treatments and potentially help develop effective vaccines against these bacteria, which in the end hopefully saves lives.

Populärvetenskaplig sammanfattning

Du består bland annat av miljontals hjältar som tillsammans skyddar dig från en mängd sjukdomar och farliga infektioner dagligen. Tillsammans utgör dem ditt immunförsvar, något som du inte hade överlevt särskilt länge utan. Immunförsvaret kan liknas med ett mycket kraftfullt och ytterst skickligt säkerhetsteam. Det finns i din kropp för att skydda dig mot skadliga inkräktare så som bakterier och virus, men också för att upptäcka cancer och rensa bort åldrade icke-fungerande celler så att de kan bli ersatta. Deras team består av många olika individer med olika tillvägagångssätt för att hålla vår kropp i balans och förgöra inkräktare. En sådan mekanism är att äta upp inkräktare och därmed döda dem. Denna process kallas fagocytos och är livsviktig i kampen mot infektioner. För att underlätta att man hittar dessa inkräktare finns det speciella taggar som markerar inkräktarna för förstörelse, så kallade antikroppar. Dessa antikroppar kan bland annat utvecklas efter en infektion eller en vaccinering som gör att immunförsvaret lätt hittar och förgör bakterien eller viruset så vi slipper bli sjuka.

Det finns dock några listiga bakterier som utvecklat sätt att undvika denna försvarsmekanism varav *Streptococcus pyogenes* är en av dem farligaste. Den är kända för att orsaka halsfluss, scharlakansfeber och ytliga hudinfektioner så som rosfeber, men historiskt sätt även varit drivande bakom dödligheten i barnsängsfeber och lunginflammationer. Än idag drabbas årligen över 700 miljoner människor och den dödar mer än en halv miljon människor varje år. Den kan orsaka livshotande infektioner där den når djupt ned i våra vävnader och in i vårt blodomlopp, så kallat invasiva infektioner. Som tur är invasiva infektioner inte lika vanliga som lindriga, men tillsammans utgör de ett stort globalt problem och man har givit den öknamnen ”mördarbakterie” och ”köttätande bakterie”. Den kan orsaka sepsis (blodförgiftning), djupa hudinfektioner så som nekrotiserande fasciit och andra allvarliga tillstånd så som förgiftningsstillståndet STSS (streptococcal toxic shock syndrome) som är mycket likt tampongsjukan. *Streptococcus dysgalactiae* är en släkting till *S. pypogenes* som också kan orsaka dessa infektioner, men har historiskt sätt inte varit lika vanlig. Tyvärr börjar även den bli allt mer vanlig, men till skillnad från *S. pyogenes* som kan drabba alla inklusive unga friska individer, infekterar *S. dysgalactiae* oftare äldre med flera sjukdomar i botten. Dess invasiva infektioner kan dessutom återkomma, vilket i princip aldrig sker med en utläkt invasiv *S. pyogenes* infektion.

I nuläget finns det inget vaccin för att skydda oss mot dessa fruktansvärda sjukdomar, utan det sjukvården kan göra är att stötta kroppen om den blir sjuk och försöka döda bakterierna med hjälp av antibiotika. I vissa mycket allvarliga fall kan man till och med behöva amputera bort infekterade kroppsdelar för att hindra infektionen från att spridas. Man kan också ge antikroppar från friska individer för att hjälpa immunförsvaret hitta bakterierna och gifterna de utsöndrar. För att vi i framtiden ska kunna utveckla vaccin och mer läkemedel mot dessa olika infektioner

behöver vi förstå bättre hur vårt immunförsvar interagerar med bakterien. Vad är det som gör att vissa kan gå runt med bakterien i halsen eller på huden utan att ens bli sjuk medan andra dör av den? Varför får vi ett långvarigt skydd efter en invasiv infektion men ytliga infektioner kan återkomma flera gånger? Mycket forskning tyder på att antikroppar som bland annat bidrar till bakterieavdödning via fagocytos kan ha en betydande roll, men mycket är fortfarande okänt framförallt kring invasiva infektioner.

Syftet med denna doktorsavhandling är att bidra med verktyg för att studera och mäta dessa interaktioner för att öka förståelsen kring vårt immunförsvars kamp mot dessa infektioner. Det är nämligen som så att det är svårt att mäta fagocytos och antikroppsfunktion på ett tillförlitligt sätt som kan jämföras mellan olika forskargrupper, över tid och mellan olika byten. I första delarbetet utvecklas en analysmetod för att mäta fagocytos som bygger på en matematisk modell (Hills ekvation) man använt inom andra områden såsom för att värdera hur effektivt ett läkemedel är. Metoden är lätt att använda och kan med små förändringar förbättra många redan tillgängliga mätmetoder. Det andra delarbetet är en modell som bygger på avancerad matematik för att förutspå hur antikroppar binder till ett mycket viktigt ytprotein (protein M) hos *S. pyogenes*. Den kan användas för att beräkna hur en tilläggsbehandling med en specifik antikroppsblandning troligen skulle bidra till immunförsvarets förmåga att markera *S. pyogenes* hos olika individer med olika antikroppsprofiler. I tredje och fjärde delarbetet används dessa två metoder för att studera om antikroppar utvecklas hos patienter drabbade av invasiv *S. dysgalactiae* respektive *S. pyogenes* infektioner. Studierna är små med få patienter men unika i sitt slag tack vare den detaljnivå och noggrannhet som vi mäter förmågan antikropparna i patienternas blod har att förbättra fagocytos av dessa bakterier.

Hos vissa patienter fann vi att fler antikroppar inte alltid innebar bättre fagocytos av dessa bakterier. Detta är viktigt eftersom det antyder att det inte bara handlar om mängden antikroppar utan också om hur bra de fungerar. Kvalitet före kvantitet helt enkelt. Detta kan vara en anledning till varför *S. dysgalactiae* kan ge återkommande invasiva infektioner. Hos patienter med *S. pyogenes* utvecklade vissa individer antikroppar som var effektiva mot flera stammar av *S. pyogenes*. Detta var en oväntad och spännande upptäckt som kan bidra till att öka förståelse för hur vårt immunförsvar arbetar för att skyddas oss långsiktigt mot fler en specifik stam av *S. pyogenes*, vilket man länge inte trott var möjligt. Tillsammans visar våra fynd hur viktigt det är att inte enbart studera vårt immunförsvars närvaro vid olika sjukdomar utan framförallt dess funktion. Vad väljer du själv? Ett säkerhetssystem som kanske är stort men som du inte vet om det fungerar, eller ett säkerhetssystem som du inte bara vet fungerar, utan dessutom hur bra det är mot olika inkräktare? I framtiden hoppas jag man kommer kunna mäta funktionen av sitt immunförsvar för att skräddarsy vilka vacciner och läkemedel man behöver och när man behöver dem. Min förhoppning är att denna doktorsavhandling kommer kunna bidra till delar av den grund som en sådan resa skulle behöva för att kunna påbörjas. En resa som

förhoppningsvis skulle vara början på något mycket större och ännu bättre än vad vi kan fantisera ihop med dagens kunskap.

Sammanfattningsvis ger denna doktorsavhandling oss nya verktyg och insikter om hur våra kroppar bekämpar vissa bakterieinfektioner. Att förstå detta kan leda till bättre behandlingar och potentiellt hjälpa till att utveckla effektiva vacciner mot dessa bakterier som i slutändan förhoppningsvis räddar liv.

List of Papers

Paper I

Therese de Neergaard, Martin Sundwall, Sebastian Wrighton, and Pontus Nordenfelt

High-Sensitivity Assessment of Phagocytosis by Persistent Association-Based Normalization

Journal of Immunology, 2021, Vol. 206, No. 1

Paper II

Vibha Kumra Ahnlide, **Therese de Neergaard,** Martin Sundwall, Tobias Ambjörnsson, and Pontus Nordenfelt

A Predictive Model of Antibody Binding in the Presence of IgG-Interacting Bacterial Surface Proteins

Frontiers in Immunology, 2021, Vol. 12

Paper III

Anna Bläckberg, **Therese de Neergaard,** Inga-Maria Frick, Pontus Nordenfelt, Rolf Lood, and Magnus Rasmussen

Lack of Opsonic Antibody Responses to Invasive Infections With Streptococcus dysgalactiae

Frontiers in Microbiology, 2021**,** Vol. 12

Paper IV

Therese de Neergaard, Anna Bläckberg, Hanna Ivarsson, Sofia Thomasson, Vibha Kumra Ahnlide, Sounak Chowdhury, Hamed Khakzad, Wael Bahnan, Johan Malmström, Magnus Rasmussen, and Pontus Nordenfelt

Invasive Streptococcal Infection Can Lead to the Generation of Cross-Strain Opsonic Antibodies

Microbiology Spectrum, 2022, Vol. 10, No. 6

Listed below are the published works during the period of my doctorate that are not included in this thesis.

Wael Bahnan, Lotta Happonen, Hamed Khakzad, Vibha Kumra Ahnlide, **Therese de Neergaard,** Sebastian Wrighton, Oscar André, Eleni Bratanis, Di Tang, Thomas Hellmark, Lars Björck, Oonagh Shannon, Lars Malmström, Johan Malmström, and Pontus Nordenfelt

A human monoclonal antibody bivalently binding two different epitopes in streptococcal M protein mediates immune function

EMBO Molecular Medicine, 2023, Vol. 15, No. 2

Lotta Happonen, Simon Hauri, Gabriel Svensson Birkedal, Christofer Karlsson, **Therese de Neergaard,** Hamed Khakzad, Pontus Nordenfelt, Mats Wikström, Magdalena Wisniewska, Lars Björck, Lars Malmström, and Johan Malmström

A quantitative Streptococcus pyogenes-human protein-protein interaction map reveals localization of opsonizing antibodies

Nature Communications, 2019, Vol. 10, No. 1

Therese de Neergaard and Pontus Nordenfelt

Quantification of Phagocytosis Using Flow Cytometry.

Methods in Molecular Biology, 2023, Vol. 2674

Abbreviations

AUC	Area under the curve
APC	Antigen-presenting cell
BCE	Before the Common Era
CD	Cluster of Differentiation
C1	Complement component 1
C3	Complement component 3
C4BP	C4b-binding protein
CFU	Colony-forming unit
CR	Complement receptor
DAMP	Damage-associated molecular pattern
DNAase	DNA endonuclease
EC_{50}	Effective Concentration, the concentration at 50 % of the maximum effect
ECM	Extracellular matrix
Endo S	Endoglycosidase S
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcR	Fc receptor
FcγR	Fc gamma receptor
FSC	Forward scatter
GAS	Group A streptococci
GCS	Group C streptococci
GGS	Group G streptococci
GTPase	Guanosine Triphosphatase
IdeS	IgG-degrading enzyme of *Streptococcus pyogenes*
Ig	Immunoglobulin
IVIG	Intravenous immunoglobulin
LTA	Lipoteichoic acid

MFI	Mean/median fluorescence intensity
MHC	Major histocompatibility
MOI	Multiplicity of infection
MOP	Multiplicity of prey
MOP_{50}	The MOP at 50 % of the maximum persistent association
NETs	Neutrophil extracellular traps
OPA	Opsonophagocytosis assay
OPKA	Opsonophagocytosis killing assay
PAMP	Pathogen-associated molecular pattern
PA	Persistent association
PAN	Persistent association normalization
PPR	Pattern-recognition receptor
PxP	Prey per phagocyte
ROS	Reactive oxygen species
SibA	Secreted immunoglobulin-binding protein from *Streptococcus pyogenes*
SL	Streptolysin
SOFA score	Sequential (Sepsis-Related) Organ Failure Assessment Score
SPE	Streptococcal pyrogenic exotoxin
SSC	Side scatter
STSS	Streptococcal toxic shock syndrome
qSOFA	Quick SOFA

Introduction

”Allt stort som skedde i världen skedde först i någon människas fantasi.”[*]

Astrid Lindgren

This thesis is about a fundamental interaction between our immune system and pathogens called phagocytosis. It is a truly fascinating defense mechanism that some pathogens have evolved diverse strategies to overcome, such as the potentially dangerous bacteria *Streptococcus pyogenes* but also its close relative *Streptococcus dysgalactiae*. In the following chapters, we will introduce several key concepts essential for understanding the themes of this thesis. We will begin with an exploration of phagocytosis and the current methods used to study it. This will be followed by a detailed introduction to *S. pyogenes*, along with a brief overview of *S. dysgalactiae*, focusing on their disease manifestations and interactions with the immune system with an emphasis on phagocytosis. Subsequently, the present investigations will be thoroughly described and discussed. The thesis will conclude with an examination of how this research may contribute to the understanding of phagocytosis quantification and invasive streptococcal infections.

[*]All the greatness in the world unfolded first in someone’s imagination.

Chapter 1: An Overview of Phagocytosis

"The pursuit of science is a grand adventure, driven by curiosity, fueled by passion, and guided by reason."

James Clerk Maxwell

Over a century ago the Russian zoologist Ilya Mechnikov discovered the role of phagocytosis in cellular immunity. Previously others had observed the process, but he advocated its significance in host response against infection.[1–3] Since then, we have come to understand its critical role not only in the defense against diseases such as infections and cancers but also in tissue homeostasis and remodeling. In this first chapter, phagocytosis will be briefly introduced.

Defining phagocytosis

Phagocytosis is an ancient receptor-driven process defined as the cellular uptake of particles larger than ≥0.5-μm. It literally means "the cellular process of eating". The cells performing this, phagocytes, were named by Ilya Mechnikov who derived it from the Greek "phagein" (to eat) and "kytos" (cell). The process itself, phagocytosis, is a term crafted by adding the suffix "oasis," indicating a dynamic cellular process.[4]

"...the intracellular uptake and digestion of foodstuff also performs a protective role against injurious agents which are either formed by the organism itself or enter the organism from outside..."[4]

Ilya Mechnikov

Phagocytic prey can be broadly categorized into two major types: those originating from the host itself, and those that are foreign, each triggering distinct responses within the immune system. Their uptake typically results in anti- respectively pro-

inflammatory responses. Those originating from the host, often referred to as altered selves, could be apoptotic bodies but also necrotic cells. The latter can result in a more pro-inflammatory response. In contrast, the foreign prey are usually microbial intruders such as viruses, bacteria, fungi, and protozoans. Phagocytes are continuously faced with the challenge of distinguishing living from aged cells, healthy from unhealthy cells, infected from uninfected cells, and pathogens from commensals. By clearing away spent cells and debris, phagocytes contribute to our tissue homeostasis, and through the eradication of pathogens and damaged cells, they protect us from disease. It is a ubiquitous process that is essential in many fields.[1,5]

The key players

Several cell types can perform phagocytosis such as epithelial cells, endothelial cells, and fibroblasts, but there is a specialized group that can do this with high efficiency, called professional phagocytes. The professional phagocytes are responsible for eradicating pathogens through phagocytosis but are also important for removing apoptotic bodies, tissue homeostasis, and regulating the immune response. Some cells, so-called antigen-presenting cells (APC) can present antigens to lymphocytes, to activate the adaptive immune response. Professional phagocytes include mononuclear phagocytes (macrophages, monocytes, and dendritic cells) and granulocytes, especially neutrophils.[1,6]

Monocytes are derived from myeloid progenitor cells in the bone marrow. They are continuously released into the bloodstream where they circulate and can migrate out into tissue upon infection or inflammation.[7,8] Furthermore, a reservoir of monocytes resides in the spleen that can be recruited during inflammation.[9] The monocytes are heterogeneous with different subpopulations and functions.[10] Traditionally they have been divided into classical and non-classical monocytes, but more recently a third intermediate subpopulation has been included. The major population of human monocytes (90 %) consists of classical monocytes, but they can also gradually develop via intermediate to non-classical.[11] They all perform phagocytosis but their gene expression differs indicating somewhat different functions. However, how much they differ and what roles they have are still under debate. The intermediate seems to be more involved in antigen-presentation, while non-classical have genes related to cytoskeleton rearrangement that can explain a more patrolling feature.[10] The classical monocytes express a broad variety of genes important for tissue repair and for sensing, regulating, and performing different immune responses including phagocytosis.[10,12,13]

Monocytes have the potential to differentiate into macrophages and dendritic cells when migrating out into tissue[14]. However, not all macrophages originate from

monocytes. Many tissue-resident macrophage populations are established during fetal development and are not dependent on monocyte infiltration.[15] Depending on the tissue the macrophages can have somewhat different functions, but in general they patrol our body for potential pathogens, clear cellular debris, and are involved in regulating inflammation. Macrophages are both important in inflammation and its resolution; during activation they undergo a process called polarization resulting in different phenotypes depending on their role. Therefore macrophages have historically been divided into pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes.[16,17] During infection, macrophages are first polarized into a pro-inflammatory M1 state with increased microbicidal activity, then the polarization becomes more towards an anti-inflammatory M2 state to aid in the tissue repairment and facilitate the resolution of inflammation.[18] This polarization is a simplification but represents the plasticity of macrophages and their diverse functions including the ability to phagocytose both microbes, cellular debris, and apoptotic bodies, and serve as APC.[19]

The dendritic cells are also positioned throughout our body and are important for sensing, collecting, and presenting antigens to lymphocytes. They originate from the bone marrow and can have both myeloid and lymphoid origins. Monocytes can differentiate into dendritic cells called monocyte-derived dendritic cells, especially during inflammation. However, there is a diversity of dendritic cells with different receptor expressions and functions. Generally, the antigen is first internalized through phagocytosis, processed into fragments and then presented at the cell surface where it is ready to interact with lymphocytes.[7,20]

Neutrophils are the most abundant leukocytes in the blood, ready to rapidly migrate to infected or injured sites where they effectively neutralize threats in various ways. They are developed in the bone marrow and belong to the granulocyte family but can also be referred to as polymorphonuclear cells because of their multilobulated nuclei.[21] In contrast to other phagocytes that have some granules, the neutrophil cytoplasm is filled with preloaded granules, which can be released upon encountering a threat. However, the granules are not only important for antimicrobial killing, but also serve as a reservoir for a variety of membrane-bound receptors in mobilizing and activating the neutrophils.[22]

The granules currently characterized are the primary (azurophilic), secondary (specific), tertiary (gelatinase) and secretory granules.[22,23] They are named in the order they are synthesized and contain different proteins. The targeting of proteins into different granules has been suggested to be dependent on the time of their biosynthesis rather than the particular protein.[24] The azurophilic granules contain antimicrobial proteins and acidic hydrolases, and are believed to be the most important granule for microbial killing. However, the specific granules still contain antimicrobial proteins but to a lesser extent.[22] The gelatinase granules and secretory vesicles are formed latest, are more easily exocytosed, and contain molecules important for adhesion and neutrophil migration.[25]

Neutrophils are crucial in the first line of defense against microorganisms and are rapid first responders with highly efficient phagocytosis. They can engulf a particle in less than 20 seconds[26] while macrophages generally require several minutes[27]. After internalization, different granules fuse with the particle-containing vesicle to neutralize and eradicate the threat.[28–30] Other means to kill pathogens is to release the granules extracellularly called degranulation[31], production of reactive oxygen species (ROS)[32] and formation of neutrophil extracellular traps (NETs)[33]. Lately, their ability to modulate the immune response and their heterogeneity under different conditions and diseases have been described.[34,35] Each type of professional phagocyte contributes uniquely to the body's defense mechanisms, collectively forming a dynamic frontline against diverse threats.[1]

The role in infections

Phagocytosis plays a crucial role in both the innate and the adaptive response to infections. Innate immunity relies on the rapid actions of professional phagocytes to engulf and neutralize pathogens. Therefore, phagocytes are dependent on the recognition of conserved surface molecules on pathogens, known as pathogen-associated molecular patterns (PAMPs). By recognizing PAMPs a broad range of pathogens can quickly be identified and targeted.[36] Some of the pattern-recognition receptors (PPRs) that recognize PAMPs can directly initiate phagocytosis such as the Dectin-1 receptor which binds to yeast polysaccharides,[37] the mannose receptor that detects mannan expressed by many microorganisms,[38,39] and the class A scavenger receptor which binds to lipopolysaccharide and lipoteichoic acid (LTA) on gram-negative respectively gram-positive bacteria[40].

Phagocytes can also identify foreign threats by recognizing humoral components that mark the prey, such as complement, which is detected via complement receptors (CR)[41] and antibodies, recognized through fragment crystallizable (Fc) receptors (FcR)[42]. This receptor-mediated threat detection exemplifies the intricate interplay between the cellular and humoral responses, as well as the innate and adaptive immune defenses.

Furthermore, antigen-presenting cells, and particularly dendritic cells, engage in phagocytosis to capture and process new microbial antigens. By presenting new antigens to lymphocytes, phagocytosis plays a pivotal role in shaping the adaptive immune landscape, ensuring a robust and tailored defense against a diverse array of infectious agents.[43]

Antibodies and their role in phagocytosis

Antibodies, also known as immunoglobulins (Ig), are essential humoral components in the adaptive immune system. They can recognize and bind to specific targets, thereby marking them for detection and subsequent engagement by various immune components, including phagocytes. Furthermore, antibodies are fundamental in establishing immune memory, enabling a quicker and more robust response upon reencountering pathogens.

Their structure consists of two identical antigen-binding fragments (Fab) that detect the same specific epitope and a Fc region (**Figure 1**). The Fc region determines the antibody's isotype and mediates its effector functions. Together, these components form a Y-shaped structure, with each arm representing one Fab fragment, and the stem constituted by the Fc region.[44] The basic structure includes two identical heavy chains and two identical light chains. The variable region of the light chain pairs with the variable region of the heavy chain to form the antigen-binding site. Antibodies can exist as monomers or further associate into higher-order structures, such as dimers, or even pentamers, depending on the specific type of antibody and its biological context. We have five different types of immunoglobulins. IgM is the first type of antibody to be produced in response to an infection, whereas IgG is produced later and provides long-term immunity. IgG is the most abundant antibody in the blood, while IgA is the most prevalent one in total, mainly located at the mucosal sites. IgE is involved in allergic reactions and parasite infections. Lastly, IgD is present on the surface of B-cells and can affect their function.[45] Moreover, IgG and IgA can be categorized into subclasses based on minor structural variations, influencing their interaction with distinct receptors and, consequently, modulating their effector functions.[46,47]

Antibodies contribute to the immune response through several mechanisms. One is neutralization, which generally occurs when antibodies bind and block the infectivity or pathogenesis of the prey, such as hindering them from infecting a host cell. Another important mechanism is complement-mediated lysis of the prey. Many of their functions, such as phagocytosis, are mediated through their interactions with FcR on different cells such as macrophages and neutrophils.[48]

Just like there are different types of antibodies there are different types of FcRs, each with distinct affinities and functions. The primary receptors for IgG are FcγRs, and they are important for antibody-mediated phagocytosis. FcγRs can be classified into two types of receptors: activating (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA) and inhibitory (FcγRIIB).[49–51]

Having briefly introduced the key players of phagocytosis in this thesis, we now transition to explore the phagocytic process itself.

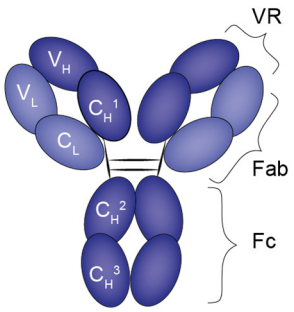

Figure 1

A schematic illustration of the structure of immunoglobulin G. The light chain (light purple) and the heavy chain (dark purple). The antigen binding site, the variable region (VR), consists of a variable heavy (V_H) and a variable light (V_L) domain. Together with a constant light (C_L) domain and a constant heavy domain (C_H) they form Fab. The Fc region consists of two constant heavy domains. The hinge is between the Fab and the Fc regions.

The process

The process of phagocytosis is complex and consists of four main phases: recognition, attachment, internalization, and digestion (**Figure 2**). First, the phagocyte needs to find and recognize the prey as a target. This is done through chemoattractants, signaling molecules, and receptor recognition of specific molecular patterns on their surface. Then, the phagocyte has to attach to the prey via specific receptors.[1] This adhesion is crucial for stabilizing the interaction and preparing for engulfment. During the internalization phase, the phagocyte membrane surrounds the target prey and forms a membrane-enclosed vesicle called a phagosome. The internalization process can occur through a range of distinct mechanisms dictated by the type of receptors, phagocytes, and prey involved. It is an active, energy-dependent process that involves the rearrangement of the phagocyte's cytoskeleton.[52] Finally, the degradation of the prey occurs in the phagosome, where the environment becomes more hostile as the phagosome matures. This is a dynamic process reliant on the fusions and fissions of different vesicles.[51]

Because phagocytosis is a receptor-driven process, it can adapt to different prey and consequently adjust the phagocytic outcome. The prey can be recognized by PPRs, opsonic receptors, and apoptotic corpse receptors. The expression of these receptors varies between different cell types and their dedicated function.[49]

When the prey is an apoptotic cell, a specific type of phagocytosis occurs called efferocytosis. It is essential for tissue homeostasis and inflammation resolution.[53,54] Apoptotic cells display "eat-me" signals like phosphatidylserine on their surface to be differentiated from healthy cells, which can express "do not eat-me" signals such as CD47 to avoid phagocytosis.[53] However, some cancer cells exploit these "do not eat-me" signals to hinder phagocytosis.[55] Multiple receptors are involved in efferocytosis, predominantly recognizing phosphatidylserine.[51] Efferocytosis promotes metabolic changes and the production of immunomodulators in macrophages, which contributes to the anti-inflammatory response.[53] In contrast, phagocytosis of necrotic cells often triggers a pro-inflammatory response. The necrotic cells might not have time or energy for controlled signaling typical of apoptosis. Instead, intracellular components are often released, many of which belong to damage-associated molecular patterns (DAMPs). These DAMPs can be recognized directly, or via the deposition of complement, by PPR and opsonic receptors, which induce phagocytosis and a pro-inflammatory response.[56]

In the following sections, the phagocytic process mediated by antibodies and complement will be described in more detail. The focus will be on FcγR-mediated phagocytosis (by macrophages) because it is the most extensively studied form of phagocytosis, and as a result, is the cornerstone of our current understanding of the phagocytic process.

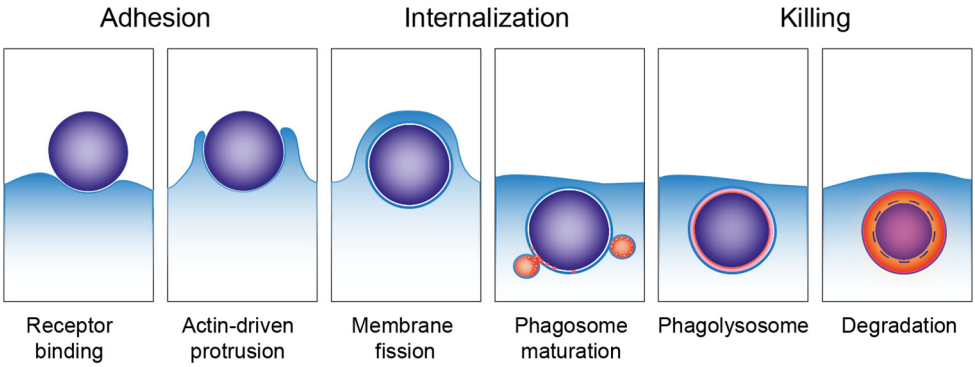

Figure 2

The phagocytic process starts with prey recognition and attachment through receptor-ligand binding. This leads to actin-driven membrane protrusions (pseudopods) creating the phagocytic cup and subsequently enclosing the prey in a membrane-enclosed vesicle called the phagosome. The internalized prey is kept in this phagosome, which matures through the endocytic pathway to a hostile phagolysosome, where the final degradation, and consequently, the killing occurs. The principle of phagosome maturation is similar for different phagocytes, however there are important differences between cell types.

FcγR-mediated phagocytosis

FcγR-mediated phagocytosis is often described by the zipper model because of the resemblance observed during the formation of the phagosome by receptor-guided local signals (**Figure 3**).[57] When IgG has bound to a prey the Fc region binds to the FcγR on the phagocyte. Through clustering of multiple FcγR, intracellular signals are triggered, leading to actin polymerization and extensions of the membrane, so-called pseudopods, around the IgG-coated prey.[58] As pseudopods surround the particle in a zipper-like fashion, a cup-shaped structure known as the phagocytic cup is formed.[52,57] The phagocytic cup is formed around the prey through serial local responses to the ligand-coated surface, eventually engulfing prey covered with IgG while half-coated prey will only result in haltered cup formation.[59] After the closing of the phagocytic cup it is eventually sealed off, resulting in the prey being within a phagosome. This phagosome is initially harmless but through biochemical modifications, it converts to a microbicidal organelle. First by fusion with early endosomes, to become early phagosome, followed by late endosomes and lysosomes resulting in late phagosome and phagolysosome, respectively.

The journey of phagosome maturation technically begins once the phagosome is cleaved from the plasma membrane. However, before the closure of the forming phagosome the fusion with endomembranes begins.[60] The maturation then involves serial fusion events with compartments of the endocytic pathway, where phosphoinositides and Rab GTPases act as crucial molecular regulators in the phagosome maturation.[61,62] The early phagosome undergoes a slight decrease in pH (ranging from 6.1 to 6.5), but when transitioning to the late phagosome it becomes more acidic (pH 5.5-6.0). This is facilitated by the acquisition of additional proton pumps.[51] The acidification continues during the formation of the phagolysosome, which is mediated by the late phagosome ultimately fuses with lysosomes. It is a highly degradative organelle, enriched in various hydrolases (proteases, nucleases, lipases) and antimicrobial peptides. For some cells, the pH can decrease to as low as 4.5-5.0.[51,62] However, this can vary depending on the phagocyte and the target. The M2 macrophages undergo rapid acidification and maturation, making them particularly effective for digesting and recycling apoptotic cells. This capability aligns with the anti-inflammatory and tissue repair roles often associated with M2 macrophages.[63] Instead, phagocytes involved in the defense against microbes such as neutrophils and dendritic cells, have phagosomes that are more close to neutral pH.[30,64,65] The less acidic environment in dendritic cells allows for tightly controlled degradation of the antigen to appropriate-sized fragments for antigen-presentation.[65] Neutrophils[66] and M1 macrophages[63] maintain a more neutral pH as a consequence of prioritizing ROS production to facilitate microbial killing.

Although both neutrophils and macrophages are professional phagocytes crucial in our defense against infections, as previously described, their operational mechanisms and characteristics are distinctively different. In contrast to

macrophages, the phagosome maturation in neutrophils is not based on the endocytic pathway, instead, it is based on the fusion of preformed granules. This contributes to the faster phagosome formation than macrophages making neutrophils extremely efficient phagocytes.[30] The order and timing of granule delivery during phagosome maturation in neutrophils are yet to be established.[30,67]

After the phagosome has completed its microbicidal and degradative activity, it needs to be resorbed. However, these aspects are almost unexplored.[62]

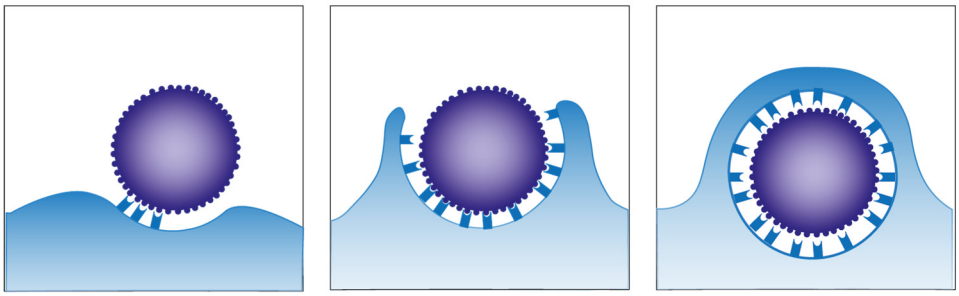

Figure 3

An illustration of the zipper mechanism that drives FcγR-mediated phagocytosis. As the prey becomes attached the receptor-ligand binding triggers receptor clustering. This leads to pseudopods form and advances around the prey as a zipper via coordinated receptor-ligand interactions, eventually resulting in the complete engulfment of the prey.

Complement-mediated phagocytosis

The complement system includes over 30 soluble proteins and is important in several immune functions including phagocytosis.[68] Activation can occur through three pathways: the classical, alternative, and lectin pathways, each initiating a biochemical cascade.

In the classical pathway, activation is triggered by the binding of the complement component 1 (C1) complex, which includes C1q, C1r, and C1s, to IgG or IgM that are attached to a prey. C1q plays a crucial role in this initial step, as it recognizes and binds to the Fc region of antibodies, thereby activating C1r and C1s, which in turn cleave the next components in the pathway. The lectin pathway is initiated by the binding of mannose-binding lectin or ficolin, which then triggers a cascade similar to the classical pathway. The alternative pathway is mechanistically different from the other two as it is activated through spontaneous hydrolysis of complement component 3 (C3).[69]

All three pathways converge on the formation of C3 convertases, leading to the cleavage and activation of C3, with the subsequent generation of C3b, which is

recognized by CR. Subsequentially, they can mediate phagocytosis, especially CR3 and CR4, which recognize the inactive form of C3b.[41,69] It is noteworthy that phagocytosis of complement-coated prey can exhibit distinct dynamics compared to FcγR-mediated phagocytosis[70,71]. Rather than pseudopods protruding from the phagocyte, the prevailing view has been that the prey undergoes a more passive sinking into the phagocyte.[72,73] However, more recent studies have revealed small actin-based membrane protrusions during complement-mediated phagocytosis, suggesting this feature is shared by different types of phagocytosis.[74,75] Still, other differences exist in the downstream signaling events.[51]

While the process may differ when studied in isolation, it is crucial to remember that in vivo, multiple receptors and cells often operate in concert, contributing to a more complex and integrated immune response.

The role of phagocytosis in this thesis

Phagocytosis constitutes a complex process involving many different cell types, receptors, and targets with unique molecular mechanisms. It is essential for many physiological processes such as development, immune response, and tissue homeostasis. Furthermore, it can also contribute to the pathogenesis of various diseases such as autoimmune, malignant, and metabolic disorders. Because of this great complexity, phagocytosis research is faced with a myriad of intriguing questions across various areas. In addition to being highly relevant for research in itself, phagocytosis is often measured as a functional outcome and can for example provide a model for studying membrane structure, receptor function, and host-pathogen interactions. As a result, it is important in many fields.

Due to its complexity, phagocytosis can be studied through a variety of methods, but can be somewhat difficult to quantify, especially in a high-throughput manner. As the field advances there is a need for a robust standardized approach to quantify and assess phagocytosis to improve the quality of data with increased reproducibility and the possibility to compare across different systems and laboratories. In this thesis, such an approach is first explored (**Paper I**) and then later applied to predict (**Paper II**), and evaluate (**Paper III** and **IV**) the antibody function during invasive streptococcal infections. This chapter has provided an overview of phagocytosis with emphasis on FcγR-mediated phagocytosis as it is essential to this thesis. In the two next chapters, we will focus on how phagocytosis can be studied and analyzed. Subsequently, we will introduce the pathogens *S. pyogenes* and *S. dysgalactiae*, and examine their intricate interactions with the immune system, emphasizing the role of phagocytosis.

Chapter 2: Methods for studying Phagocytosis

"Perfection is the enemy of good."

Voltaire

In science, it all starts with a question or hypothesis. Carefully defining it can be challenging, but valuable when planning how to answer it. After all, determining how the data shall be acquired, how to set up the experimental system, and how to analyze the results depends almost entirely on the question being asked. This chapter will present an overview of methods that can be used to study phagocytosis and some important in vitro considerations. The following chapter will describe different approaches to analyzing the resulting phagocytosis data.

Phagocytosis assays

Studying phagocytosis can be challenging since the phagocytic process itself is by nature complicated and dynamic. Therefore there are many different potential aspects to explore. As a result, numerous methods have been developed to address the various aspects of phagocytosis. However, we still lack the methods to fully capture this process.

Phagocytosis assays can be divided into direct and indirect assays. Direct assays measure the interaction between the phagocyte and prey, while indirect assays measure components that are correlated with the process such as the presence of a protein or biochemical alterations.

Studying phagocytosis of apoptotic[76,77], necrotic[78], and tumor cells[79] is often done through direct assays by fluorescently labeling the target cells or model prey, but may sometimes be conducted through indirect tests[80,81]. Conversely, the study of phagocytosis involving microbial prey, especially bacteria, has historically relied more on indirect assays and continues to play a significant role in current research.

Bacterial killing has been viewed as the end result of phagocytosis and therefore it has been common to employ killing assays to study phagocytosis. Bacterial killing is still seen as one of the primary and desired outcomes of phagocytosis, but other factors can affect bacterial killing, even during phagocytosis. Several pathogens, such as *S. pyogenes*, *Staphylococcus aureus*, *Listeria monocytogenes,* and *Mycobacterium tuberculosis* to mention only a few, have evolved the ability to survive intracellularly and resist killing. These strategies include escaping from the phagosome, inhibiting its maturation, or resisting degradation, thereby evading the usual immune response of the phagocyte.[49] Furthermore, professional phagocytes have many other strategies to kill bacteria such as degranulation, oxidative burst, and formation of NETs.[6,21,82]

These bactericidal tests evaluate the outcome of a reaction between bacteria and phagocytes, and sometimes other blood components as well. Often these results are attributed to phagocytosis, but one cannot be certain since they do not measure it directly. In this thesis, they are therefore referred to as indirect phagocytosis assays when used to study phagocytosis, but direct bactericidal tests when used as such.

Bactericidal test: an indirect phagocytosis assay

The great strength of these bactericidal tests is that they actually measure the often desired outcome of phagocyte-pathogen interactions. This can be of particular importance when studying special interventions, the humoral response in general, potential phagocyte-mediated protection, or the end results of phagocytosis.[83]

Traditionally the Lancefield assay, described by Rebecca Lancefield in 1957 as the "indirect bactericidal assay", has been used.[84,85] Whole human blood is the source of phagocytes for both direct and indirect bactericidal assays. In the direct test, antibodies are supplied from the same blood, whereas in the indirect test, they can be supplied from other sera or plasma sources, such as rabbit or human. Regarding the indirect test only non-immune blood can be used as a phagocyte source, otherwise, antibodies other than those of interest might accidentally be introduced.[85] This presents a significant challenge, as natural immunity to *S. pyogenes* is widespread among many potential donors, thereby limiting their suitability for use. Furthermore, it necessitates a robust definition of non-immune blood to ensure the validity of the assay. To address this issue and minimize donor variability, a modification of this assay has been developed where the donor blood is treated with the IgG-degrading enzyme IdeS (as detailed in Chapter 4), effectively reducing the impact of pre-existing antibodies in the assays.[86] Another alteration to reduce the variability in the Lancefield assay is the use of phagocytic cell lines instead of primary phagocytes.[83]

Whether using the original Lancefield assay or any of its modifications, the assessment of the bactericidal effect is consistent: after incubating the phagocytes

with the bacteria, the effectiveness is measured through the inhibition of bacterial growth. This is commonly done by manually counting the number of colony-forming units (CFU) on plates after overnight incubation. However, it can be laborious, therefore alternatives such as turbidity, radiation, and chemiluminescence measurements, as well as automated colony counters have been developed.[87–90]

The logic behind these assays is that a dead bacterium cannot grow thus less growth indicates more killing. Consequently, this method is only suitable for culturable viable prey.[85] Complicating it further, growth arrest has been shown to sometimes be uncoupled from bacterial killing[91]. In addition, if used to primarily study phagocytosis rather than killing, prey that can survive intracellularly may give a false impression that no phagocytosis has occurred, thereby missing important information. This might sometimes have been the case for *S. pyogenes,* as it was initially believed to be strictly extracellular[92].

Variants of these methods are often used in order to evaluate the opsonic capacity of antibodies and are then called opsonophagocytosis assays (OPAs), sometimes specified as killing OPAs or opsonophagocytosis killing assays (OPKAs). They have been shown to correlate with protection for pneumococcal vaccines and are therefore a laboratory gold standard in pneumococcal vaccine studies.[93] Efforts have been made to develop similar assays for *S. pyogenes*[84], but they have faced criticism regarding reproducibility. Furthermore, the failure of some strains to perform effectively in these assays, coupled with their inability to predict protection, further supports the need for additional assays.[94]

In summary, these methods can be used for evaluating the end result of the microbe-humoral-phagocyte interaction, provided that careful attention is given to ensure reproducibility. However, these assays do not offer insights into the details of the phagocytic process itself, for such analysis, more direct approaches are necessary.

Direct phagocytosis assays

Direct assays are those studying the phagocytic process, or a part of it, where the prey is recognized, attached, and finally engulfed by the phagocyte (**Figure 4**). If successful this will lead to the destruction of the prey. The fundamental feature of the techniques used to study phagocytosis directly is the ability to separate phagocytes with prey from those without. This can quite easily be achieved by many different techniques, commonly through labeling the prey and/or phagocyte, followed by the use of microscopy or flow cytometry. However, a major challenge in evaluating this process is distinguishing between prey attachment to the cell surface and internalization by the phagocyte [95]. As a consequence, this is not a feature shared by all phagocytosis assays. However, depending on the question being asked, this is often essential in order to understand the process.

The data acquisition techniques for direct observations are mainly done via different types of microscopes[96], flow cytometers[97], or a combination of both; imaging flow cytometry[98]. These often include steps to separate the attached prey from internalized by different labeling techniques, commonly with fluorescence, or by removal of the extracellular prey through washing or, if the prey is a bacterium, by killing via antibiotics. Another viable method to examine internalization is through phagosome isolation[99,100].

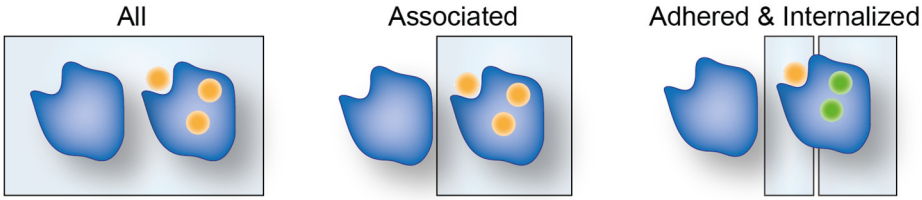

Figure 4

Phagocytes' interaction with prey can be studied by merely assessing a total interaction (all) or defining the interaction by distinguishing between phagocytes with prey (associated) from those without. The association can be further determined by separating adhered prey from internalized.

Data acquisition techniques

Principles of fluorescence

Fluorescence is a phenomenon in which a substance absorbs light of a particular wavelength and then re-emits it at a longer wavelength. The molecules that are capable of this phenomenon are called fluorophores. When a fluorophore absorbs photons of light at its specific excitation wavelength, it promotes an electron to a higher energy state. The electron then releases energy in the form of light and emits photons of a longer wavelength (lower energy because some is lost to heat) when it returns to its ground state. The difference between these wavelengths is known as the Stokes shift (**Figure 5**).[101] Flow cytometers and fluorescence microscopes use lasers to excite fluorophores, and can then detect and amplify the emitted light. Some fluorophores are pH-sensitive while others are stable, which can be utilized to differentiate between the acidic environment in the phagosome compared to the often more neutral one outside the phagocyte[102]. This can also be achieved in some cases by deliberating quenching the fluorescence of external prey.[95,103] A particular strength of fluorophores is the multitude of things they can label. For example, they are often conjugated with biomolecules such as antibodies, used in specific dyes, or introduced to the cells in the form of fluorescent proteins. With their versatile

applications, fluorophores play a crucial role in studying phagocytosis both through microscopy and flow cytometry.

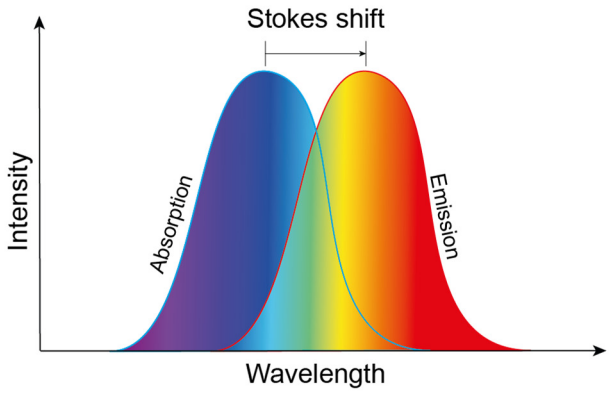

Figure 5

Illustration of Stokes shift. The graph features two distinct peaks: the first peak represents the absorption of photons at a shorter wavelength (higher energy), while the second peak represents the emission of photons at a longer wavelength (lower energy). The difference between these two peaks (Stokes shift) highlights the energy loss that occurs as the absorbed light is re-emitted at a lower energy level.

Microscopy

Light microscopy, a cornerstone in the world of biological research, has the advantage of providing direct observation of study objects at a single-cell level with quantitative spatial information often at subcellular resolution. It can be used to monitor dynamic processes live, but also a halted process where the samples have been fixated, enabling detailed labeling with fluorescent dyes and antibodies.[104]

Data acquisition often relies on manual decisions, making it time-consuming, and therefore seldom high-throughput, in addition to the risk of unwanted observer bias and reduced reproducibility. Unlike flow cytometry, it is challenging for microscopy to characterize the whole sample population while maintaining high resolution. However, recently methods for automated data acquisition and data analysis using computer-assisted microscopes have been developed with the potential to address these issues.[105,106]

Flow cytometry

Flow cytometry is another powerful technique used in many fields to analyze the characteristics of individual cells within a population. By passing single cells in a fluid stream through laser light, it can measure various properties, including size,

granularity, and other characteristics, such as expression of specific proteins, through fluorescent markers.[107] It is a high-throughput technology that can provide single-cell information and identify subpopulations within the context of the sample population.[108] Unlike microscopy, the sample is consumed during data acquisition, preventing its use for live monitoring of dynamic processes within a sample. Furthermore, this technique does not allow for the spatial quantification of subcellular components with high resolution.[109] Hence, it is often used as a high-throughput screening method and then later combined with detailed microscopy. However, there are flow cytometers available already combining these two techniques called imaging flow cytometers.[109] Perhaps not as high resolution as fluorescent microscopes, but for phagocytosis, they are often sufficient and adds the strength of a flow cytometer[110].

When the laser light hits the particle it produces both scattered and fluorescent light, which is collected by detectors through filters with different wavelengths. The light is then converted into data that can be analyzed by a computer. Visible light scatter is not affected by fluorescence and is measured in two different directions: forward scatter (FSC) and side scatter (SSC) at 90°. FSC correlates to the relative size of the cell, and SSC correlates with the granularity of the cell.[108] By using flow cytometry cell populations can be studied based on their fluorescent or light-scattering features.

Naturally, in phagocytosis assays the prey is generally smaller than the phagocyte, which is a relevant feature for the data acquisition. Similar to microscopy, distinguishing between the phagocytes and prey is usually done easily through size and granularity, but can be improved with labeling. However, when the sample includes different cells, as in phagocytosis assays, the flow cytometry requires the smaller cell to be fluorescent in order to separate interacting cells from non-interacting. The principles of separating prey attachment from internalization are the same as for microscopy except that the spatial resolution is lost, therefore some sort of fluorescent labeling is also necessary for this purpose.[97]

Both flow cytometry and microscopy allow for the analysis of multiple parameters simultaneously.[109] However, due to spectral overlap and limitations in the number of available fluorophores, the number of parameters that can be measured in a single experiment is still restricted.[107] For general phagocytosis assays this is seldom a problem because it is often enough to fluorescently target the prey with one or two fluorophores to be able to determine association and/or internalization.[97,102,110] Therefore, it is possible to avoid spectral overlap through careful consideration of fluorophores. This is one of the many methodological considerations to be made when studying phagocytosis in vitro, a topic we will briefly review next.

In vitro considerations

In vitro, which translates from Latin to "in glass", refers to studies conducted outside the study object's normal biological context, typically in a controlled laboratory environment, while in vivo studies are conducted within the living organism. In vitro studies not only form the foundation of phagocytosis research today but also constitute the methodology used throughout this thesis. These studies offer the advantage of providing a controlled and precise environment, often essential for understanding intricate biological processes. However, the simplicity of in vitro studies represents a double-edged sword, as the complexity present in vivo is lost. Furthermore, this meticulous control over the experimental conditions necessitates careful consideration of various factors to ensure the relevance and reliability of the findings.

The phagocyte

Using primary cells has the great advantage of studying real phagocytes. Since the primary cells are purified from donor blood the usage of these requires special ethical consideration. Due to their natural heterogeneity both between and within individuals, they can introduce unwanted variability to an in vitro system[111]. Furthermore, both neutrophils and monocytes are short-lived and cannot easily be genetically manipulated in comparison with immortalized cell lines.[112]

Therefore immortalized cells with phagocytic features similar to those of primary cells are viable alternatives as model phagocytes. There are several different phagocytic cell lines, human-derived such as the (pro)monocytic cell lines THP-1[113] and U-937[114], and the promyelocytic cell line HL-60[115], and from other origins such as the murine macrophage cell lines RAW 264.7[116] and J774A.1[117].

The promyelocytic cell line HL-60 can be differentiated into cells with phenotypes more like neutrophils, monocytes, or macrophages, whereby lose their ability to proliferate.[118] The monocytic cell lines, as well as the primary cells, can be differentiated into cells similar to various types of macrophages or dendritic cells. Differentiation to macrophages makes them adhesive and no longer grow in suspension.[119,120]

THP-1 cells

In this thesis, the primary source of phagocytes has been the THP-1 cell line. It is a human monocytic cell line derived from peripheral blood from a 1-year-old boy with acute monocytic leukemia in 1980 by Shigeru Tsuchiya.[113] These cells serve as a valuable model for studying various aspects of immune functions. In phagocytosis studies, THP-1 cells are widely used since they express multiple FcγRs even without differentiation.[121,122] The expression of FcγRI (CD64) and FcγRII

(CD32) is not upregulated after differentiation;[122,123] however, other phagocytic receptors are. Compared to their less or undifferentiated counterparts, differentiated THP-1 cells generally show higher overall phagocytic activity, driven by many different types of receptors.[121,124] This means that undifferentiated THP-1 cells have a lower phagocytic activity baseline from these other receptors, leading to a more pronounced relative signal change when Fc-mediated phagocytosis occurs.[121] Therefore, undifferentiated THP-1 cells are a good model phagocyte for studying Fc-mediated phagocytosis. Furthermore, undifferentiated THP-1 cells grow in suspension, making them particularly convenient in flow cytometric-based assays. Margaret Ackerman together with colleagues has shown undifferentiated THP-1 cells express FcγRI, both FcγRII subtypes: activating IIA and inhibitory IIB receptors, but also FcγRIII (CD16), making this cell line suitable when studying the effects of a broad range of FcγRs.[121] The complement receptors are primarily expressed after differentiation into macrophages[123] and dendritic cells[125]. Nonetheless, it is important to remember that THP-1 cells are not primary cells and, therefore, may not fully capture the complexity of nature. Despite this limitation, their ease of culture and amenability to high-throughput assays make them valuable complements to primary cells and in vivo studies in phagocytosis research, potentially contributing to insights into the intricacies of phagocytosis.

The prey

Another important methodological consideration is of course what type of prey to use. This is highly dependent on the research question, but also the practical aspects and safety concerns. Live prey can be crucial for some questions, but for many others, alternatives such as killed or model prey can be more suitable. There are multiple model preys that can be used such as latex beads[126,127], red blood cells (often sheep)[126], and zymosan particles (based on the cell wall components of yeast)[128,129]. Red blood cells have a relatively homogeneous size and are rarely internalized in their existing state providing a low phagocytic baseline. These features, along with the ability to remove extracellular cells through osmotic lysis, make them advantageous for phagocytosis assays. Furthermore, their easy detectability under a light microscope is a useful characteristic in microscopy-based assays. However, red blood cells can be deformed during phagocytosis resulting in the risk of underestimation.[126] Latex beads on the other hand are uniform, hard to destroy, available in various sizes, and are commonly fluorescent to facilitate detection through microscopy and flow cytometry.[126] They can often be coupled with various molecules, which can offer meticulous control over the receptors and ligands involved in the phagocytic process.[127,130] As a result, we know that the size and shape of the prey affect phagocytosis[131–133], but also, the size and density of the ligands[130,134].

Between the model prey and the real live prey, there is the killed prey. Similar to beads, killed prey are metabolically inactive. As a consequence, factors produced by a live prey cannot be captured[135], but the surface structures and characteristics of the prey may be studied[136]. For killed prey in contrast to beads, these structures will consist of a more complex structure with multiple ligands and therefore hopefully share more resembles with the live prey. However, there is a risk that the killing itself might affect these characteristics.

There are different techniques to metabolically inactivate a bacterium including ultraviolet irradiation, heat-killing, antibiotics, and paraformaldehyde fixation.[137] For killing *S. pyogenes,* heat-killing at 80 °C for 5 min followed by rapid cooling is a well-established protocol, but may have to be altered depending on the bacteria[135].

Coating the prey with various molecules is another crucial aspect of the prey's condition. When the prey is coated with an opsonin it is called opsonization. The term "opsonin" is derived from Greek and means "I cater for" or "I prepare victuals for".[138] The name is appropriate as an opsonin is a molecule that prepares the prey for phagocytosis by making them more "appetizing" for phagocytes. These opsonins, often antibodies or complement proteins, attach to the prey's surface, enhancing recognition, attachment, and internalization by the phagocytes. Depending on what type of phagocytosis to study different opsonins will be suitable, for Fc-mediated phagocytosis incubating with specific antibodies is sufficient. Incubation with serum or plasma also involves immunoglobulins, but compared to exclusive antibody opsonization, it is more complex and allows for more physiological opsonization. Furthermore, the contribution of the complement can be removed through heat inactivation. For complement-mediated phagocytosis, an additional stimulus is needed to activate the different pathways.[126,139] If opsonization is not performed, the surface ligands on the prey will likely interact with receptors on the phagocytes, potentially leading to phagocytosis. However, controlling for the specific receptors involved may be challenging. Moreover, the microenvironment plays a crucial role in the prey-phagocyte interaction. Therefore, not only the selection of appropriate opsonins but also the correct concentration and potential mixtures of other biomolecules are important.[136,140]

As previously mentioned, different techniques are commonly utilized to detect prey that are being internalized and separate them from the solely attached ones. Fluorescent antibodies targeting the extracellular prey[97,110], quenching the extracellular signal[95,103], and the usage of pH-sensitive dyes[102] that emit fluorescence in the acidified phagosome are some possible techniques. It is advisable to add control samples with impaired internalization such as incubation on ice or actin-inhibitors.[95,141] In addition to all the biological aspects of the prey to consider, other more practical ones are essential as well and those relevant to phagocytosis will be reviewed in the following section.

External factors

Beyond the biological factors that affect phagocytosis, there are several physical and experimental factors that can impact phagocytosis assays. Many of these factors were described by Eric Ponder in 1927 in a review about physical factors affecting phagocytosis.[142] He begins with the following:

> *"As it is impossible for a cell to ingest a particle unless the two first come into contact, we have in the first place a series of factors which regulate the frequency of such collisions; these factors are principally those of the size, concentration, and relative movement of the cells and particles. Not every such collision, however, is followed by phagocytosis of the particle by the cell,.."*[142]

He continues to review the potential physical factors that can alter the ingestion rate after a collision has occurred but with no satisfactory results. Instead, he elegantly poses the question of whether the purely physical hypothesis is sufficient as an explanation.[142] Almost 100 years later, we now know that there are other factors, such as biological ones, that are central to phagocytosis. Nevertheless, many of the physical factors he described remain relevant. Some of them are essential to control, particularly those related to collision frequency since the internalization is partly dependent on the rate of collisions.[142]

The factors affecting the collision rate in a solution can be derived from collision theory. In designing phagocytosis experiments, many of these factors are relevant, including volume, time, temperature, and the concentrations of both prey and phagocytes.[143]

In **Paper I** we evaluated the impact of these factors on the outcome of phagocytosis and proposed a method to normalize their effect, aiming to enhance the quality of phagocytosis data.[144] This method is based on assessing a range of concentrations of the prey per phagocyte ratio, or the multiplicity of prey (MOP), a term introduced by us in **Paper I.** The idea behind the term was that we needed a more general term than the commonly used multiplicity of infection (MOI), which is only applicable when the prey is an infectious particle such as in the infection biology field. Hence, this thesis will use MOP.

The experimental factors previously mentioned are relevant for any type of phagocytosis assays, and many of them can alter the dynamic range of the system, typically addressed through optimization. To aid with optimization, **Paper I** offers a systematic standardized approach to phagocytosis assays as a guide to the dynamic range of the system. However, when determining the experimental set up the practical aspects of performing these experiments should be taken into account as well. In summary, numerous non-biological factors influence phagocytosis, but once identified they can be effectively controlled.

Chapter 3: Analyzing Phagocytosis

"To tell you the truth we don't do it because it is useful but because it's amusing."

Archibald Vivian Hill

As mentioned in the previous chapter, depending on your scientific question at hand, different parts of the phagocytic process may be more relevant to assess. Whether it is the first interaction, the internalization process, or the eradication of the prey that is of interest, your experimental system and data assessment need to be set up accordingly. Here, we will focus on the initial steps of phagocytosis and how to analyze them in different ways.

The phagocytic process from an analytic perspective

The first level of experimental resolution, association, is sufficient for most questions and therefore typically used when studying phagocytosis. In this thesis, association is defined as the persistent interaction between a prey and a phagocyte, with the prey either internalized or adhered to the phagocyte. To assess association the system must be able to discriminate between phagocytes without prey from those with prey. Usually, this is done by labeling one of the cells, which is most often the prey for convenience.

If the system can determine whether a prey is outside or inside a phagocyte, an assessment of adhesion and internalization can be performed. This can be achieved through different approaches, aforementioned in Chapter 2. The terminology here is of importance because even if both association, adhesion, and internalization are often called phagocytosis in the end, it can be beneficial to be more precise with the terminology when analyzing the data. One can easily imagine two different scenarios; one where adhesion has increased but not internalization, and one in which the reverse effect has occurred. Both of these scenarios result in enhanced phagocytosis, but the underlying biological mechanisms are likely to differ.[145] In this thesis, the term interaction is used in a general sense, applicable to any part of the process.

The team or the star

Independent of which part of the process is being studied, the phagocytic capacity of the phagocytes can be analyzed both at an individual and at a population level. To visualize this, one can view the phagocyte population as a sports team with the goal of internalizing as much prey as possible as quickly as possible. If half of the team is internalizing prey, one way to improve the outcome is to improve the overall capacity of the team by increasing the number of phagocytes internalizing prey. The phagocytic capacity has increased at the population level. To eradicate the same number of prey as the previous example but instead improve the phagocytic capacity at an individual phagocyte level, the ability of individual team members has to improve. In other words, phagocytes already internalizing a prey must internalize even more prey.

This mental exercise not only provides entertainment but has also been shown to be relevant when studying phagocytosis. For some patients with chronic myeloid leukemia, the total number of neutrophils increased during a relapse. However, the additional neutrophils only exhibited low phagocytic activity, and no increase in numbers was observed in neutrophils with high phagocytic activity.[146] In a study with healthy individuals ingesting glucose compared to overnight fasting, the overall phagocytic ability of their neutrophils decreased without altering the total count.[147] The importance of assessing phagocytosis both at the population and individual phagocyte level can be exemplified further with the physiological changes in healthy pregnant women where neutrophil count is increased but the individual phagocytic ability is reduced.[148,149] Moreover, various prey, particularly pathogens, possess mechanisms that can influence either the overall ability of the phagocyte population to engage or their individual capacity to internalize these targets. Specific examples of such interactions are detailed in Chapter 4. Consequently, to excel in the sports of phagocytosis, one must have a team full of intermediate players or a team with at least one true star player doing all the work. However, in biology, it is probably often a combination that affects the phagocytic ability, therefore, it may be relevant to quantify both.

At the population level, the data is often reported as the percentage of phagocytes interacting, while at the individual phagocyte level, it is rather the signal intensity or the number of prey an average phagocyte is interacting with.

Quantifying phagocytes and prey

The advantages of image-based assays are several, and a great one is the ability to directly count the number of prey and phagocytes studied either manually or with image software.[145] In non-image-based systems such as scintillation counters or flow cytometry the quantification is not as straightforward. However, the signal

intensity measured (fluorescence, radiation) has been shown to correlate well with the count.[90,141,150,151]

In flow cytometry, the phagocytes are usually big enough to be detected on the characteristics of size (FSC) and granularity (SSC), previously described in Chapter 2. This is often sufficient for prey detection and quantification as well when studied separately. However, when they interact, only the bigger cell of the two will be possible to quantify using these characteristics alone. To identify the smaller cell additional characteristics, such as fluorescence signal intensity, can be utilized. Therefore fluorescent labeling of prey is commonly used as described in Chapter 2, but the signal intensity can vary depending on the fluorophore and its batch, the prey, and different flow cytometers.

This experimental variation can be addressed by expressing the signal intensity of prey to a comparable reference point. One such point is the number of prey per phagocyte; in **Paper I**, we abbreviate it to PxP. By determining the average fluorescent signal intensity (MFI) of a single prey unit such normalization can be made. This is done by measuring the MFI of samples containing only free prey. The number of prey interacting with the phagocyte may then be calculated by dividing the prey-MFI emitted by the phagocyte with the MFI of a single prey. Noteworthy, depending on the prey, the smallest prey unit may not always be a single prey, instead, it can consist of a small chain containing several cocci, as in the case of streptococcal bacteria. In other words, by normalizing the signal intensity of the prey relative to a reference point for example MFI of single prey, the data becomes more consistent and easier to compare across different systems.

The prey perspective

Phagocytosis is the interaction of two different key players, the phagocyte and the prey. As a researcher one can, thus, originate from at least these two different perspectives. The previous sections describing the different aspects of assessing phagocytosis have been from the phagocyte perspective. However, most of them are applicable also when coming from a prey perspective. For example, association can be expressed in how much of the prey population has become associated with phagocytes. The data acquisition needs to be able to capture all the prey in the sample, but if so, the portion of prey interacting with a phagocyte can be analyzed in the same manner as described for the phagocyte. Similarly, it can be applied to adhesion and internalization as well.[98]

Phagocytic indexes

Analyzing and reporting each part of the phagocytic process can be a bit cumbersome. As a way to condense their data and improve comparability,

researchers have over the years come up with different types of indexes to report overall phagocytic capacity. In 1975 Bianco et al. introduced a phagocytic index based on the percentage of phagocytes interacting with prey multiplied by the average number of prey interacted per phagocyte.[70] The index was criticized for not taking into account whether the prey was attached or internalized. By adding this information Nordenfelt et al. introduced another index called phagocytic ability. The phagocytic ability index is calculated by taking the ratio of phagocytes that interact with at least one prey and multiplying it by the internalization fraction. This internalization fraction is determined by the ratio of intracellular prey to the total number of prey per interacting phagocyte.[145] Ackerman et al. modified the phagocytic index by Bianco et al. to create a phagocytic score when the exact number of prey was unknown. Instead, the prey MFI was used as the factor to multiply by the percentages of phagocytes interacting with prey, also called integrated MFI[152]. A few years later, the same research group, led by Galit Alter, changed the term to phagoscore. Furthermore, they began to use the area under the curve (AUC) as an additional parameter, which will be described in the upcoming sections.[121,153] Others use the term phagocytic index when solely reporting the number of prey ingested per phagocyte[147,148,154–157], while it has also been applied to describe the total MFI signal of internalized prey for a whole sample[158].

The many available phagocytic indexes together with the lack of shared terminology and standards highlight the challenges the field is facing, especially in comparability and reproducibility of results. In **Paper I** we try to address these problems by introducing a systemic approach to assess phagocytosis and suggestions for standardized terminology.

The math of dose-response curves

The Hill equation was first described in 1910 by Archibald V. Hill to explain the equilibrium relationship between oxygen tension and the saturation of hemoglobin[159]. Since then, the Hill equation has been widely used to describe many different biological, physiological, and pharmacological relationships.[160,161] The original equation was a three-parameter equation with two variables, a dependent one (y) and an independent one (x). The three parameters considered were: y_{max}, c, and the coefficient α. See Equation 1.

$$y = \frac{y_{max} x^{\alpha}}{c^{\alpha} + x^{\alpha}} = \frac{y_{max}}{1 + \left(\frac{c}{x}\right)^{\alpha}} \qquad (1)$$

Another well-known equation is the Michaelis-Menten equation, introduced 1913 and was based on the work of Victor Henri. It can be described as a special example of the Hill equation applied to the relationship between the velocity of a reaction and the concentration in a single substrate enzyme reaction with the coefficient α set as 1. Noteworthy, the velocity is a dynamic effect compared to the static effects that are classically described by the Hill equation, however, both can be viewed as a reaction at equilibrium.[160,162,163]

By introducing a baseline parameter (Y_0), the y value when x equaled zero, the Hill equation became a 4-parameter equation. This model is also commonly referred to as the 4-parameters logistical or the E_{max} model, especially in pharmacological studies where the relationship between drug effect (E) and drug concentration (C) are quantified[164,165]. Often the two variables x and y are substituted by C and E, respectively. Consequently, Y_{max} is replaced by E_{max}. The parameter c is replaced by EC_{50} which is the inflection point of the curve and stands for the half-maximal effective concentration. The coefficient, also known as the Hill coefficient, Hill constant, or Hill factor, determines the steepness of the curve. It has several different abbreviations such as N, d^h, n^H and α, henceforth n^H will be used.[160,164,166] By setting the baseline to zero, the previous equation can be rewritten as the E_{max} equation following the substitutions, as shown in Equation 2.

$$E = \frac{E_{max}}{1 + \left(\frac{EC_{50}}{C}\right)^{n^H}} \qquad (2)$$

The curve generated from this equation is typically sigmoidal with the steepest part in the middle, generally known as a dose-response curve (**Figure 6**). The x-axis is often logarithmic. On the y-axis, if the maximum effect (E_{max}) can be estimated, then the effect (E) can be expressed as a ratio or percentage of E_{max} permitting the normalization of the y-axis.[160] This type of equation should only be applied on data where only one inflection is observed. Alterations to this model have been suggested when assessing data with several inflections.[165]

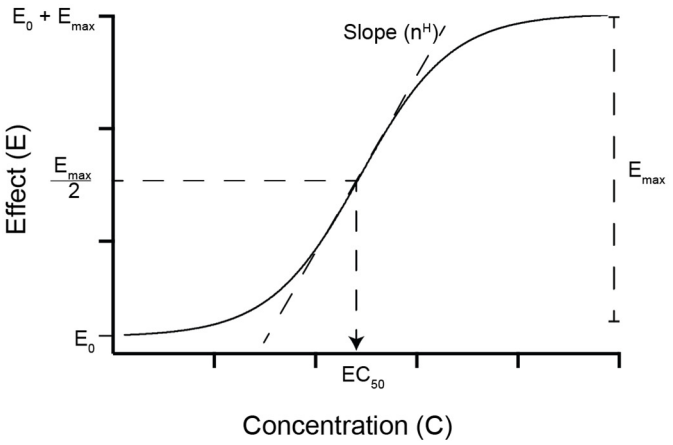

Figure 6

A dose-response curve of the E_{max} model, where effect (E) is the response and concentration (C) is the dose. The E_{max} is the maximum response elicited and the top value is the sum of the baseline (E_0) and E_{max}. EC_{50} is the dose required to elicit half of the E_{max} and the Hill constant (n^H) determines the steepness of the curve.

The different parameters

E_{max} is the maximal effect size that can be attributable to the drug/stimuli/agonist alone, while E_0 is the baseline response without that stimulus. The effect size is the range between the top value (upper asymptote) and the curve's starting point. If E_0=0 then the top value and E_{max} are the same. However, the top value of the curve is the sum of E_0 and E_{max}. This is relevant when evaluating responses with multiple stimuli at the same time.[164,166]

EC_{50} is the concentration where 50 % of the maximal effect is seen, correspondingly EC_{10} is at 10 % of E_{max} and EC_{90} is at 90 % of E_{max}[164]. This may reflect the sensitivity in the system and/or the affinity in ligand-receptor interactions[160,161]. A decrease in the EC_{50} value will result in a left curve shift. The relationship between laws of equilibrium and the Hill equation was described by Alfred J. Clark 1933 among others[167]. Similarly to EC_{50}, parameter c from the original equation (1) is directly linked to the equilibrium dissociation constant K_D from the law of mass action, $c = K_D^{(1/\alpha)}$. However, when considering the possibility that intermediate complexes can be formed, the modeling becomes more complicated. Therefore, K_D is used when the binding, rather than the effect, of ligand-receptor interactions is quantified.[160,161,164,168]

The Hill constant determines the steepness of the curve and therefore gives the model flexibility for a good data fit. In addition, it can provide insights into the heterogeneity of the response and the cooperativity of ligand binding. In cases where the Hill constant is greater than 1, it becomes easier for the next ligand to bind after the first one has bound. This happens because the affinity increases, as in the case of oxygen binding to hemoglobin where the first molecule induces conformational changes that improve the affinity of the following molecules. On the other hand, if the Hill constant is less than 1, negative cooperativity occurs, resulting in changes that reduce affinity. When the Hill constant is equal to 1, independent binding happens, and the affinity is not affected by whether or not other molecules bind. In scenarios with extreme positive cooperativity, such as with oxygen and hemoglobulin, an estimation of the number of binding sites can be derived from the Hill constant. However, this estimation is only a minimum estimate and more binding sites can occur.[160,161] In pharmacology, the Hill constant can reveal a specific characteristic of a drug effect. For antibiotics, it has been used together with E_{max}, to describe if the killing is concentration- or time-dependent[160,169]. When studying the pharmacodynamics of HIV-1 drug resistance, the Hill constant and IC_{50} (the inhibitory version of EC_{50}) have been used to describe how different mutations affect the antiviral activity.[170]

The AUC is a single numerical value to summarize the overall performance and is not limited to only dose-response curves. AUC can be calculated on a sample series, for example, time or MOP. It represents the total response and can indicate the overall efficacy. AUC can simplify the evaluation of a curve by condensing its information into a single value. The AUC of the prey signal emitted by the phagocytes in a sample series can be used as a cumulative measure of the number of prey either associated or internalized over the specified variable such as MOP, antibody titers or time.[141,153]

Dose-response curves in different fields

As one can imagine, the flexibility and generalization of the model (or similar expressions) have resulted in many applications in a number of fields, particularly pharmacology[171]. Alfred J. Clark was the first to use the Hill equation in pharmacology during his study with acetylcholine in 1926[167,172]. For example in physiology, the Hill equation has been used to describe aminoglycoside-related nephrotoxicity[173], voltage-dependent ion channels[174], enzymatic reactions[162,163], and oxygen with hemoglobulin[159]. However, at the time **Paper I** was published no one had used the Hill equation to describe the interaction between a prey and a phagocyte in the aspects of phagocytosis[144].

Chapter 4: Phagocytosis in Streptococcal Infections

”Små sår och fattiga vänner ska man inte förakta.”[*]

The intricate interplay between hosts and microbes is fascinating. While the majority occur in symbiosis and are essential for host functions, some are lethal. A particularly interesting microbe is the human pathogen *S. pyogenes*, which is involved in a wide variety of interactions causing everything from asymptomatic carriage to deadly invasive infections. Each year, it is the cause of 700 million mild skin and throat infections and 600,000 invasive, potentially life-threatening ones. In this chapter, the group A streptococcus *S. pyogenes* will be introduced, starting with its disease manifestations and virulence factors. Many of these are shared with *S. dysgalactiae*, another beta-hemolytic streptococcus belonging to group C or G, and will be presented in more detail at the end of this chapter.

The diseases

For thousands of years, *S. pyogenes* has been infecting humans. In the late 5th century BCE (Before the Common Era), Hippocrates described disease manifestations later associated with *S. pyogenes* such as puerperal (child-bed) fever, erysipelas, and necrotizing fasciitis as a complication of erysipelas.[175,176]

> *“Many were attacked by the erysipelas all over the body when the exciting cause was a trivial accident or a very small wound; especially when the patients were about sixty years old and the wound was in the head, however little the neglect might have been. Many even while undergoing treatment suffered from severe inflammations, and the erysipelas would quickly spread widely in all directions. Flesh, sinews and bones fell away in large quantities... there were many deaths.”*[176]

[*]Small wounds and poor friends should not be overlooked.

The different disease manifestations can be categorized as invasive, noninvasive, and post-infectious sequelae. However, the fourth infectious state is asymptomatic, with no disease manifestations present.

Invasive infections have an annual incidence of approximately 2.45 per 100,000 in developed countries and even higher in less developed ones. Globally, it is the cause of more than 160,000 deaths each year. This includes but is not limited to necrotizing fasciitis, sepsis, puerperal fever, and streptococcal toxic shock syndrome (STSS).[177,178]

Necrotizing fasciitis is well described in the quote by Hippocrates and is characterized by soft-tissue death and often multiorgan failure. A good descriptive term commonly used by the public is "flesh-eating disease". In cases of necrotizing fasciitis involving monomicrobial infections, group A streptococcus (GAS), along with group C or G streptococci (GCS/GGS), is the major causative agent, responsible for more than 80 % of the infections.[178–180]

STSS was first reported by Cone et al. in 1987[181] and is characterized by early onset of shock and multiorgan failure, mostly due to a toxin-induced cytokine storm similar to toxic shock syndrome caused by *Staphylococcus aureus*.[182–184] It is often accompanied by necrotizing fasciitis, but not exclusively.[185] *S. pyogenes* is a common cause of STSS but other streptococci, such as GCS/GGS, can also cause this condition.[186,187]

Historically, puerperal fever was a leading cause of maternal infection and mortality, often caused by *S. pyogenes* infecting the uterus.[188] Other common pathogens today are *Escherichia coli* and group B streptococci, but sometimes *S. pyogenes* and GCS/GGS can be the cause.[189,190] In modern times the mortality rates can be as low as 2 % in some reports, but peripartum infections due to *S. pyogenes* are still associated with worse outcomes and higher morbidity than other pathogens.[189,191]

Sepsis, also generally known as blood poisoning, is a life-threatening condition where tissue and organ damage is caused by the body's response to an infection. The definition of sepsis has been reevaluated multiple times based on evolving knowledge of pathophysiology, diagnostics, and severity. Initial definitions were developed in a consensus conference 1991[192], and were last reexamined in 2016[193] with an altered definition of sepsis and septic shock, the so-called Sepsis-3 criteria. The authors define them as follows:

> *"Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection…Septic shock is defined as a subset of sepsis in which underlying circulatory and cellular metabolism abnormalities are profound enough to substantially increase mortality."*[193]

Sepsis can affect different vital organs and functions such as respiration, coagulation, liver, renal, cardiovascular, and central nervous system. SOFA score, Sequential (Sepsis-Related) Organ Failure Assessment Score, is a scoring system to aid in identifying and clinically characterizing septic patients and their organ dysfunction. It is based on alteration in above mentioned vital functions.[193,194] In addition, a bedside score, called quick SOFA (qSOFA), has been developed to assist clinicians in recognizing patients with sepsis or the risk of developing it. The score is based on alterations in mental status, systolic blood pressure, and/or respiratory rate.[193] Still, these invasive infections can in some cases be difficult to distinguish from the less invasive ones, and sometimes they even originate from a non-invasive infection or possibly an asymptomatic carriage, especially with *S. pyogenes* infections.[179]

Pharyngitis is a very common non-invasive *S. pyogenes* infection, also generally known as sore throat. It mostly affects children between 5 and 15 years old and is normally self-terminating. Scarlet fever is a characteristic rash that can accompany *S. pyogenes* infections, usually pharyngitis, but has become less common due to penicillin treatment.[195] Other superficial infections include erysipelas and impetigo, both infecting the superficial layers of the skin. Impetigo infects the outermost layer of the skin which results in crusty lesions, whereas erysipelas affects somewhat deeper and is therefore characterized by a red rash with a clear demarcation to non-infected tissue. *S. pyogenes* is the primary streptococci causing these types of infections, but GCS/GGS have also been demonstrated to be responsible.[196]

Rheumatic fever, including rheumatic heart disease, and acute post-streptococcal glomerulonephritis are immune-mediated disorders typically developed after an *S. pyogenes* infection. Acute post-streptococcal glomerulonephritis seldom results in permanent renal damage, however, rheumatic fever is a major contributor to acquired heart disease in children worldwide.[184,197] Together these post-infection sequelae cause a significant burden to morbidity and mortality globally, especially in children.[177] The molecular mimicry between streptococcal and human antigens is believed to be a contributing factor in the development of these diseases. Especially some types of the streptococcal M protein have been found to share epitopes with human tissue of the heart and kidneys. In rheumatic fever recurring streptococcal infections are also believed to play an important role in disease development.[184]

It is primarily children that can have asymptomatic carriage of *S. pyogenes*, typically in the posterior pharynx. The prevalence in schoolchildren is around 10-20 %[198–200]. There is evidence supporting different expressions of virulence traits between carriage and symptomatic infection such as capsule production and intracellular survival. However, it is yet to be determined which bacterial and perhaps even host factors are important for asymptomatic carriage to occur.[200]

S. pyogenes can infect immunocompetent and previously healthy humans. However, it mostly infects children and adolescents.[201] Other risk groups are the

elderly and individuals with comorbidities, but also pregnant and post-partum women.[201,202] The transmission occurs primarily through respiratory droplets or in direct contact with an infected person but also via contaminated material.[201]

The primary treatment of these different types of infections is antibiotics, often penicillin, but depending on severity broad-spectrum antibiotics, supportive intensive care, surgery, and intravenous immunoglobulin may also be necessary. Despite these interventions, it is sometimes not enough.[203] Furthermore, at the time of writing, no vaccine against *S. pyogenes* infections is available even after great efforts have been made by the community. Given the global burden *S. pyogenes* infections cause, this emphasizes the importance of continuing the quest to understand this pathogen and its interaction with our immune system.

The pathogen *S. pyogenes*

S. pyogenes is a gram-positive beta-hemolytic streptococcus, meaning that they are round cells (coccus, berry in Greek) often growing in chains (strepto, chain in Greek), and on blood agar plates they cause destruction of the red blood cells. The name *pyogenes* was introduced by Friedrich J. Rosenbach in 1884 and means pus-producing in Greek.[204] There are several different beta-hemolytic streptococci, therefore in 1933 Rebecca Lancefield introduced a classification system based on specific carbohydrate surface antigens and subdivided them into different groups depending on their origin.[205,206] *S. pyogenes* belongs to group A since it infects humans, and GAS is most often used synonymously with *S. pyogenes*, including in this thesis. However, the group A antigen can sometimes be expressed by other streptococcal strains such as *S. dysgalactiae*. *S. dysgalactiae* is also a beta-hemolytic streptococci and causes similar infections as *S. pyogenes*, but most often carries group C or G antigen, and therefore typically belongs to GCS/GGS.[206] Lancefield further divided GAS into different serotypes based on the antigenic variability of a surface protein named M[85,207] which was later determined to be a major virulence factor for *S. pyogenes*.

Interactions with the immune system

S. pyogenes has developed a wide array of different strategies to infect and colonize the human body successfully. These virulence factors contribute to its pathogenicity through multiple mechanisms necessary to establish and preserve an infection, including evading the immune defense. The expression and importance of different virulence factors can change throughout an infection and between strains. In the following section some key virulence factors will be briefly introduced.

M protein

A central virulence factor for *S. pyogenes* is the aforementioned surface protein M, which has an important role in many different immune evading mechanisms, including but not limited to adhesion, invasion, and resistance to phagocytosis[208]. It is an α-helical coiled-coil protein extending out 50-100 nm from the cell wall in the form of hairlike structures.[136,208] M protein typically forms a dimer and is encoded by the *emm* gene.[209]All *S. pyogenes* possess an *emm* gene but there is great variability between different M proteins reflecting that more than 200 different *emm* genotypes have been identified.[210] The M protein consists of two regions, one variable, and one more conserved region. The N-terminal faces the external environment with a hypervariable part at the end which contributes to the evasion of the immune defense.[208] It is the region where type-specific antibodies bind, which is what determines the serotype of a strain and is therefore the region assessed during *emm* typing.[211] In contrast, the more conserved part is closer to the bacterial surface. The M protein consists of different repeating sequencing units called A, B, C, and D. A is located closest to the N-terminal followed by B and C, while D is located nearest the C-terminal.[208] Based on what repeats the *emm* type consists of, different *emm* pattern groups have been determined called pattern A-C, pattern D, and pattern E (**Figure 7**). The pattern A-C has the longest M proteins with a bigger variable portion compared to the other two groups. Globally, the majority of the identified strains belong to either pattern D or E and are together responsible for a significant disease burden.[210] About 20 % of the different *emm* types belong to the pattern A-C group, and these are usually associated with throat infections. Pattern D strains are more commonly isolated from superficial skin infections such as impetigo while pattern E affects both sites.[210,212] Therefore, it seems that different strains prefer different tissues to infect, and some strains are more associated with invasive disease than others. The *emm*1 strain belonging to the pattern A-C has been the dominant type associated with invasive infections in Europe and North America in the last decades.[213,214] It is still unclear what contributes to the differences in disease manifestations and tissue tropism between the different *emm* patterns, but associated genes and the M protein itself, including its structure and size, have been suggested.[210,212]

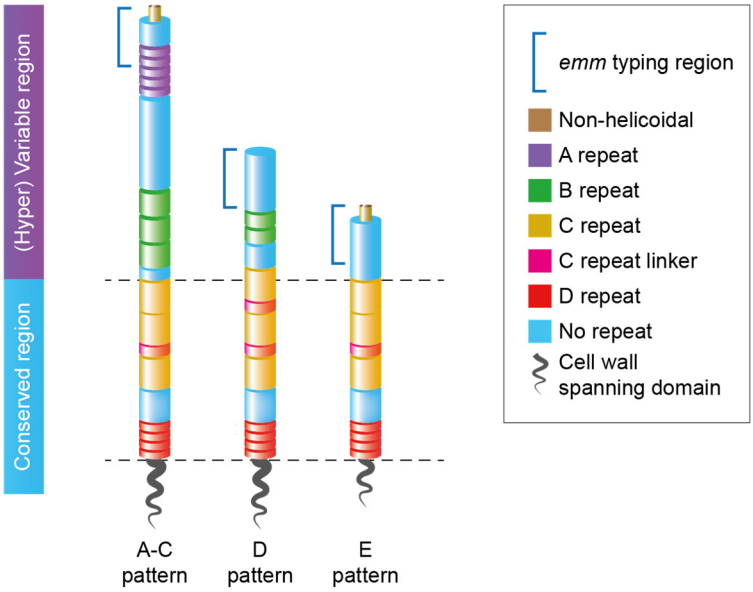

Figure 7

This figure illustrates the structural variations among three M protein types: M5, M80, and M77, representing the three different *emm* pattern groups, A-C, D, and E respectively. The M protein consists of two regions, one (hyper)variable, and one more conserved region. M proteins from pattern A–C *emm* types are characterized by longer structures, with a longer variable portion, while patterns D and E have shorter variable portions. Notably, A repeats are largely absent in M proteins from patterns D and E. B repeats are commonly found in patterns A–C and D, but are typically missing in pattern E *emm* types. The conserved regions of these proteins show more similarity; D repeats are highly conserved, and only minor variations exist between the C repeats. Each C repeat unit comprises of 35 conserved residues, occasionally separated by a seven-residue C repeat linker. Approximately 20 % of M proteins, such as M80, lack a non-helical N-terminus. Figure adapted from McMillian et al. 2013. [210]

Adhesion and invasion

S. pyogenes colonization starts with the pathogen attaching to a mucosal or cutaneous surface. There it has to compete with the normal flora and several mechanical forces such as salivary and mucous fluid flow, and epidermal exfoliation.[184] The adhesion process has been described as an initial weak interaction followed by a more tissue-specific and stronger adherence. This process is dependent on a large repertoire of different adhesins, but they are all based on the principle of bridging the distance between the pathogen and the host. The first attachment step is mediated, among others, via LTA by reacting with surface molecules of *S. pyogenes* such as the M protein, and with fibronectin on human epithelial cells and serum albumin.[215,216] This complex may also be involved in

biofilm formation which helps the pathogen withstand hostile environments.[217] Another adhesin important for the longer-distance interaction is the pili, which are long, flexible rods extending up to 3 μm from the cell surface.[218] The second stage of adhesion occurs more closely with more specific interaction through protein-protein or glycan-protein interaction with the cell surface or the extracellular matrix (ECM). *S. pyogenes* expresses multiple different fibronectin-binding proteins important for adherence to the ECM, but can also bind other components in the ECM such as collagen and laminin. The M protein itself has been shown to contribute to adhesion by binding different host ligands including collagen, sialic acid, and membrane cofactor protein (CD46), but its role in direct binding to host cells is not yet clear.[216] Furthermore, the expression of specific adhesins might vary between different serotypes, tissue sites, and local environments. Moreover, many of the adhesins, including the M protein, can also contribute to the invasion of the epithelial cells. This intracellular invasion provides shelter for the pathogen from the surrounding environment and the immune system.[219]

Enzymes and toxins

S. pyogenes has numerous virulence factors that are secreted with enzymatic and/or toxic activity. The characteristic beta-hemolytic effect is caused by the hemolytic exotoxin streptolysin S (SLS). SLS damages the cell membranes of multiple host cells including polymorphonuclear leukocytes. This cytotoxic capacity is shared with Streptolysin O (SLO).[219] SLO also works synergistically by translocating a second toxin, NAD glycohydrolase, into the cytosol of host cells which triggers cell death.[220] The secreted virulence factors do not only induce damage to the host cells, but also provide protection for the streptococci. For example, enzymes that can degrade DNA, so-called DNAases, protect *S. pyogenes* from being killed by NETs[221]. The DNAase may also contribute to bacterial dissemination together with other virulence factors such as streptokinase and streptococcal pyrogenic exotoxin B (SpeB).[219] Streptokinase forms a complex with plasminogen, the zymogen of plasmin, and through conformational changes activates the human serine protease plasmin. This can occur with free, but also bacterial surface-bound plasminogen. Plasmin is able to degrade fibrinogen clots, connective tissue, and the ECM, which is important in bacterial spreading.[222] SpeB is a broad-spectrum protease known to cleave more than 200 proteins, among others important for the ECM, inflammation, and the immune system including immunoglobulins.[223,224] Other pyrogenic exotoxins are the superantigens. The first ones were isolated from patients with scarlet fever and were shown to induce fever and increased susceptibility to endotoxic shock when injected in rabbits.[225] Currently, thirteen streptococcal superantigens have been reported.[226] SpeA and SpeC were the first ones to be described together with SpeB, however the latter later proved not to exploit superantigen activity. The superantigens share the ability to induce excessive stimulation of T cells resulting in massive release of cytokines. Unlike normal

activation, unprocessed superantigens can bind simultaneously to major histocompatibility (MHC) class II molecules on APCs and the T cell receptor on T cells resulting in a large unrestrained activation.[184,226] This cytokine storm can contribute to the hypotension and multiorgan failure characteristic of severe GAS infections, particularly STSS.[226,227] The amplitude of the response has been shown to differ between individuals and might be one explanation for varying severity during *S. pyogenes* infections.[228] Another pro-inflammatory virulence factor is the M protein in soluble form, particularly the M1 protein has been well characterized. When M1 is cleaved from the bacterial surface it can activate platelets, neutrophils, monocytes, and T cells subsequentially inducing a strong inflammatory response.[229–231] The potency for T-cell activation has been similar to superantigens, and therefore some suggest M1 protein can be viewed as a superantigen.[232]

Avoiding phagocytosis

In order to successfully infect, invade, and survive within a human, *S. pyogenes* has evolved multiple strategies to resist phagocytosis and its subsequentially killing. These virulence factors include complement inhibitors, leukocidal toxins (aforementioned SLO and SLS), and modulators of the immunoglobulins effect.[233] Several *S. pyogenes* strains can express a hyaluronic acid capsule creating a physical barrier against antibody-binding[234] and complement deposition[235] resulting in resistance to phagocytosis. Another anti-phagocytic strategy is to bind fibronectin through fibronectin-binding proteins or via the M protein, however, the exact mechanism responsible for this is still unknown.[140]

The complement system is important for the host in the defense against pathogens. Consequently, it becomes crucial for *S. pyogenes* virulence to control the complement system, and as a result, the pathogen has evolved multiple strategies to inhibit it. The M protein can bind fibrinogen and the regulatory C4b-binding protein (C4BP) to inhibit C3 convertase reducing complement-mediated phagocytosis through the classical pathway[236–240]. Moreover, the alternative pathway can be inhibited by M protein binding to the complement regulator factor H and factor H-like protein 1 which are important for C3b degradation on the bacterial surface.[236,241,242] In the complement-mediated inflammatory response, C5a is an important chemotaxin to recruit polymorphonuclear leukocytes and mononuclear phagocytes to the site of infection. This has *S. pyogenes* taken advantage of when inhibiting C5a activity via its C5 peptidase resulting in a decreased number of phagocytes and their bacterial clearance at the site of infection.[243,244] It is a complex system *S. pyogenes* possess to inhibit complement and the expression of these inhibitors varies between *emm* types.

In addition to the complement system, the immunoglobulins are fundamental in facilitating phagocytosis and bacterial killing, as previously described in Chapter 1. There are several mechanisms *S. pyogenes* uses to interfere with how

immunoglobulins bind to their surface in order to evade their effect. The immunoglobulins bind to the pathogen via their Fab fragments exposing the Fc region to interact with Fc receptors on the phagocytes. The IgG-degrading enzyme of *S. pyogenes* (IdeS) specifically targets and cleaves human IgG just below the hinge region resulting in $F(ab')_2$ fragments lacking Fc domains. The $F(ab')_2$ fragments can still bind, however, the effector functions mediated through the Fc domain, such as complement activation and FcγR-mediated phagocytosis, can no longer occur.[245,246] In a similar manner, the *S. pyogenes* enzyme endoglycosidase S (Endo S) specifically cleaves the glycan from the Fc region of IgG, which also affects the effector functions of IgG.[224,247,248]

Another strategy *S. pyogenes* uses to block the Fc function is the ability of the M protein to bind the Fc region of IgG (**Figure 8**). This binding results in a protective reversed antibody-binding orientation with the Fab fragments faced toward the external environment. This reversed binding occurs mainly in low concentrations of IgG such as saliva, while in higher concentrations such as plasma, the immune-mediated Fab-binding prevails.[136,249]

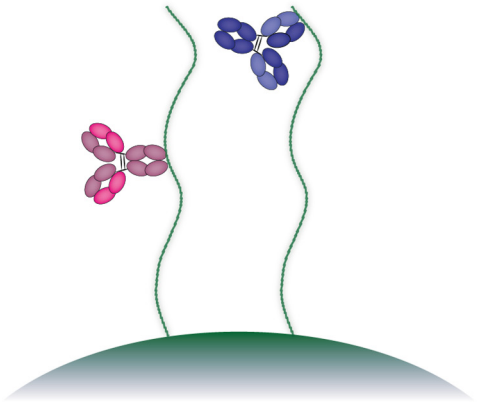

Figure 8

Schematic illustration of two antibodies binding to the M protein in two different orientations, via Fab (purple) in an immunogenic manner and via the Fc region (pink) in a non-immunogenic manner.

In addition to the M protein, *S. pyogenes* expresses other immunoglobulin-binding proteins that can bind IgG or IgA, and sometimes both.[250,251] Most of them are surface proteins, but there also exists the secreted immunoglobulin binding protein from *S. pyogenes* (SibA) that can bind both IgG, IgA, and IgM.[252]

Moreover, if *S. pyogenes* becomes internalized it possesses additional virulence mechanisms to survive intracellularly such as impairing the acidification of phagosomes and adapting metabolically. Since *S. pyogenes* can survive inside

phagocytes where it is well-protected from the external hostile environment sometimes it utilizes this as bacterial reservoirs.[253–256]

Immunity against *S. pyogenes*

Acquired immunity against *S. pyogenes* takes many years to develop, a phenomenon largely attributed to the pathogen's diversity, particularly its multitude of *emm* types.[257] Rebecca Lancefield was the first to describe the type-specific immunity conferred by antibodies targeting the hypervariable region of the M protein.[205,258] These type-specific antibodies have been reported to persist for up to 30 years and remain capable of facilitating type-specific bacterial killing.[258,259] The generation of these antibodies usually starts about 4 weeks after an infection.[260] However, a study by Bisno et al. in 1974 indicated that children with skin or throat infections do not always develop a type-specific immune response.[261] Furthermore, it has been proposed that in instances of superficial skin infections, multiple exposures might be required for the persistence of type-specific antibodies, as demonstrated in a mouse model.[257] This might partly explain why recurrent superficial infections are relatively common, whereas repeated invasive *S. pyogenes* infections are rare.[262]

In both human cases and mouse models of invasive *S. pyogenes* infections, antibodies targeting both the hypervariable and the conserved regions of the M protein have been observed. Notably, while antibodies against the conserved part tended to dominate the immune response, only the type-specific antibodies demonstrated the capacity for passive immunization.[263] Additionally, the presence of type-specific antibodies has been correlated with decreased susceptibility to invasive infections, although they do not appear to influence the severity of these infections.[264] Furthermore, early research found evidence of only type-specific antibodies to be opsonic. Consequently, there is a prevailing belief that only antibodies targeting the hypervariable region of the M protein are protective against *S. pyogenes*.[265]

Recent developments in vaccine research have challenged this traditional view. Studies have reported that antibodies targeting the more conserved binding sites on the M protein can also exhibit opsonic activity.[266–268] In collaborative efforts not included in this thesis, our findings support this newer perspective. For instance, in one study, we determined that the C-terminal, but not the N-terminal, of the M5 protein was crucial for opsonization.[246] In another study, we discovered an anti-M antibody naturally developed after a superficial *S. pyogenes* infection that possesses opsonic capacity across multiple *emm* types.[269] These findings, along with observations of cross-opsonic antibodies in children following superficial skin infection[270], suggest the possibility of broader immunity mechanisms conveyed by

antibodies. Nonetheless, research on immunity against *S. pyogenes* remains a field with many unanswered questions, warranting further in-depth studies.

The cousin *S. dysgalactiae*

S. dysgalactiae also possesses many of the virulence factors *S. pyogenes* has including the M protein.[271] Some can express a similar enzyme as Endo S[272]and many can bind immunoglobulins in a non-immune manner via the Fc-binding protein G[273]. They often cause similar diseases, but where *S. pyogenes* can infect young previously healthy individuals *S. dysgalactiae* has been viewed more as an opportunist more often infecting the elderly with comorbidities.[271,274] However, in recent years the incidence of invasive *S. dysgalactiae* infections has increased significantly with a 30-day mortality rate of around 15 %.[275,276] In contrast to invasive *S. pyogenes* infection,[262] *S. dysgalactiae* regularly causes recurrent invasive infection in 4-10 % of the cases[271,274,276,277]. Little is known about how immunity is developed after *S. dysgalactiae* infection and the exact mechanism behind these recurrent infections is unknown. But most certainly it is an interplay between many different host and bacterial factors. The failure to develop immunity after an invasive infection is intriguing, and understanding it may not only be relevant for *S. dysgalactiae* infections, but might shed light on the immunity for other invasive streptococcal infections such as *S. pyogenes* as well.

The pathogens' role in this thesis

The strategies *S. pyogenes* and *S. dysgalactiae* have evolved to survive in the hostile environment created by our immune system are impressive. Consequently, it is not surprising that the presence of antibodies alone does not guarantee protection. Not only can they degrade our antibodies, but they can also alter the orientation of the antibody binding in a non-immune fashion. Understanding the molecular driving force behind this behavior for *S. pyogenes* and predicting the orientation of antibody binding was the purpose of **Paper II**. This is essential for advancing our comprehension of the intricate host-pathogen interaction and the development of a protective immune response.

The development of protective immunity to *S. pyogenes* and *S. dysgalactiae* infections is not completely understood. For many decades we have known that type-specific immunity can be generated naturally after an *S. pyogenes* infection. Nonetheless, the development of a broader immunity would be preferable, but whether this can occur naturally remains unknown. Furthermore, the difference in recurrent invasive infections between the two streptococci raises the question of

how immunity may be developed after severe infections. In **Paper III** and **IV**, we therefore studied the development of antibodies and their opsonic capacity in patients during invasive *S. dysgalactiae* respectively *S. pyogenes* infections.

The Aim

"The important thing is not to stop questioning. Curiosity has its own reason for existing."

Albert Einstein

The overall aim of this thesis is to develop a universal and robust method for quantifying phagocytosis and subsequently apply it to study the opsonic capacity of antibodies in invasive streptococcal infections.

The specific aims of each paper:

Paper I

To develop a universal and robust method for quantifying phagocytosis.

Paper II

To develop a biophysical model to describe and predict the competitive binding between antibodies and the streptococcal M protein.

Paper III

To investigate the antibody response in patients with invasive *S. dysgalactiae* infection.

Paper IV

To investigate the opsonic capacity of antibodies in patients with invasive *S. pyogenes* infection.

General methods and methodological considerations

"An expert is someone who has made all the mistakes that can be made in a very narrow field."

Niels Bohr

In Chapter 2 different methodological approaches and considerations are presented. Each method comes with its own strengths and limitations, because no experimental system is perfect. In general, throughout this thesis, the phagocyte used has been THP-1 cells. One exception is a supplementary experiment with neutrophils as primary cells, in **Paper IV**, where big donor variability was seen. The cell line has been a consistent, homogenous, and highly available source of phagocytes.

The prey has varied from beads to both gram-positive and gram-negative bacteria, laboratory strains, and clinical isolates. However, with a few exceptions all bacteria have been heat-killed and later sonicated to disperse large aggregates. The primary reason for this has been to increase the safety of conducting these sometimes tedious experiments, especially when handling clinical isolates known to have caused severe invasive diseases. Furthermore, together with the sonication it allows us to control the MOP and reproduce it more consistently. The drawback is of course the loss of many of the prey's virulence factors and the risk of altering the surface molecules that antibodies target. Still, it can be advantageous for some questions to isolate the interaction and solely focus on the receptor-antibody-antigen interaction without any other factors disturbing. In **Paper I** we saw a difference with higher association for heat-killed *Escherichia coli*, but no big difference in internalization for those phagocytes engaging in phagocytosis. Therefore, we did a comparison in **Paper IV** between live and heat-killed bacteria with no obvious difference detected for neither association nor internalization.

All prey were fluorescent, either from start or labeled by us, for most assays a double labeling was performed to be able to distinguish between adhered and internalized prey, either through pH-sensitive fluorophores or with secondary antibodies. Samples incubated on ice were used consistently as an internalization control.

Data acquisition was almost exclusively performed through flow cytometry, but for **Paper I** an imaging flow cytometer was used as a validation of the data acquisition. However not published, fluorescent microscopy was used in the beginning to confirm labeling and to evaluate which data acquisition technique to use. The reason why flow cytometry became the final choice was it provided high-throughput quantifiable data, which was not obtained as easily from the fluorescence microscope at that time.

Phagocytosis has primarily been assessed through direct assays because we have been interested in the initial phase of phagocytosis rather than the end outcome as measured through bactericidal tests. However, in **Paper III** the antibody-mediated bacterial killing was of interest, and consequently investigated with a bactericidal test. There the phagocytosis assay served as a complementary method to augment the bactericidal assay, allowing a more comprehensive capture of the phagocytic process.

The study designs throughout this thesis have been streamlined to reduce experimental variation and increase the detection of small biological differences. This comes at the cost of complexity and physiological relevance. However, it is often necessary when addressing research questions at a molecular level. In the specific cases of **Paper III** and **Paper IV**, a limited amount of clinical samples were available for assessing the opsonic capacity of the antibody response. Clinical samples are naturally heterogeneous, and this can often be managed with larger study populations. The small study population is, therefore, a major limitation in these two studies, while their strengths lie within the detailed assessment of phagocytosis. Something that would not have been feasible with bigger study populations. Consequently, by focusing on the detailed assessment of opsonic capacity, the relevance is evident, and the studies can offer illustrative instances. However, due to the limited sizes of our study populations, it is advisable to exercise caution and avoid drawing general conclusions.

Ethical statement

The ethical considerations in this thesis mainly involve **Paper III** and **IV** with human participants. All participants provided their written and oral informed consent to participate in the respective study. Their involvement entailed one or two additional blood samplings and sometimes sampling for bacterial culturing. Both of these procedures can be perceived as unpleasant but are associated with very to extremely low risks. Furthermore, the participants attended a follow-up meeting with a physician involved in the research project. Their participation in the study did not affect the medical care they received. The potential benefits from the studies include an improved understanding of the antibody response during invasive

streptococcal infections, which might contribute to the prevention and treatment of such diseases.

For **Paper III** (2016/939) and **Paper IV** (with the amendment 2018/828), the Regional Ethics Committee of Lund University reviewed and approved the involvement of human participants in respective studies.

Present investigations

"If there is effort, there is always accomplishment."

Jigoro Kano

Paper I: High-sensitivity Assessment of Phagocytosis by Persistent Association-Based Normalization

The complexity of phagocytosis is not limited to the process itself but also includes the different parameters to consider when studying phagocytosis. Despite phagocytosis assessments being important in many fields accurate quantification is still a challenge. After a literature search, we could conclude there is currently no gold standard or established methodology for robustly comparing phagocytosis data across different experiments, systems, and laboratories. This lack of a standardized approach, combined with the multitude of available phagocytic indexes and the absence of shared terminology, highlights the difficulties faced by the field in achieving comparability and reproducibility.

In an effort to address these challenges in **Paper I**, we first had to determine what factors can affect phagocytosis apart from biology itself. Derived from collision theory and the work by Eric Ponder described in Chapter 2 we examined several experimental factors' effect on the outcome of phagocytosis. Experimental factors that increase collision frequencies such as increased MOP, decreased reaction volume including centrifugation, and increased reaction time all increased the phagocyte-prey association, called persistent association. This association itself resulted in more prey being associated with the individual phagocyte even if there were no biological differences between the conditions. The persistent association was directly coupled with internalization both on a population and individual phagocyte level indicating experimental factors may affect internalization as well. Furthermore, we showed that the relationship between persistent association and MOP could be described by the Hill equation, introduced in Chapter 3.

In the present work, the Hill equation was introduced as a method to assess phagocytosis and to use when persistent association normalization (PAN) is required. In the 4-parameters logistical (E_{max}) model, the persistent association (PA)

is the dependent variable, and MOP is the independent one (Equation 3). Consequently, the inflection point is based on calculating the MOP ratio needed to elicit half of the phagocytes to associate persistently. We termed it MOP_{50} which is equivalent to EC_{50}. The MOP_{50}-value itself can be used to quantify the ability a phagocyte population has to persistently associate, with lower values indicating a more effective population. Therefore, similar to EC_{50} in other fields, it can be used to compare different conditions and systems.

$$PA = \frac{PA_{max}}{1 + \left(\frac{MOP_{50}}{MOP}\right)^{n^H}} \quad (3)$$

Furthermore, at MOP_{50}, additional investigations into phagocytosis details, such as the quantification of prey adhered and internalized per phagocytes, can be conducted with normalization for factors influencing persistent association, including experimental ones (**Figure 9**). As a proof of principle, we showed that the effect the experimental factor volume had on phagocytosis could be normalized using PAN. At MOP_{50} there was no longer a difference in the number of prey associated with an individual phagocyte for the different volumes. We conclude that experimental factors influencing persistent association can be normalized at MOP_{50}. As a consequence, when comparing PAN to standard ways of assessing phagocytosis it provides increased robustness, sensitivity, and reproducibility of the data.

In Chapters 2 and 3, different types of phagocytosis assessments available were introduced. In **Paper I** we summarize them in an effort to contribute to consistent terminology and shared definitions, and subsequently add PAN as a complement. It can be incorporated into most existing phagocytosis assays by assessing phagocytosis over a range of MOPs. In addition, it can serve as a guide to the dynamic range of the system and indicate at which MOP detailed studies should be conducted.

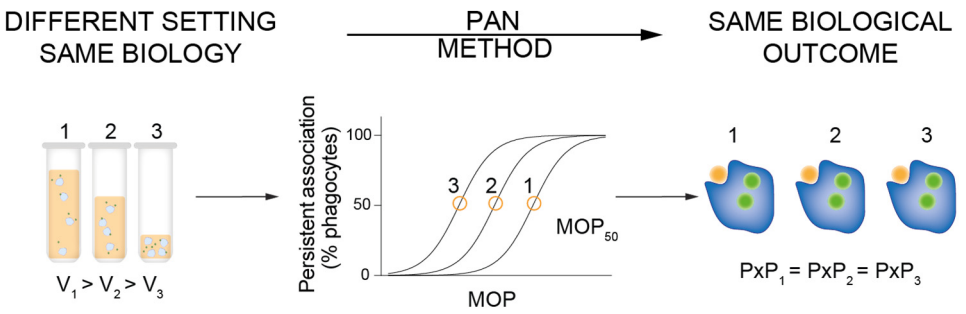

Figure 9

An illustration of the hypothesis upon which PAN is based: if experimental factors' impact on persistent association, such as volume, can be normalized at MOP_{50}, the same number of prey per phagocyte (PxP) should be detected when biology is consistent. Here, a decrease in volume results in higher persistent association at the same MOP, causing a left shift in the curves, but the biological outcome will be the same at the same level of persistent association, in other words, at MOP_{50}.

In the present work, PAN was applied to different biological systems and allowed us to compare distinctive types of prey from different experiments with different conditions. Exemplifying its potential to provide comparable assessments cross-laboratory, across different biological systems, and over time. Furthermore, translating phagocytosis data into a mathematical function provides additional parameters to evaluate (**Figure 10**). Whether these parameters mean anything mechanistically for phagocytosis is not yet known, but by using the Hill equation when analyzing phagocytosis data curve characteristics can be quantified and compared with the potential of revealing biological insights not accessible before.

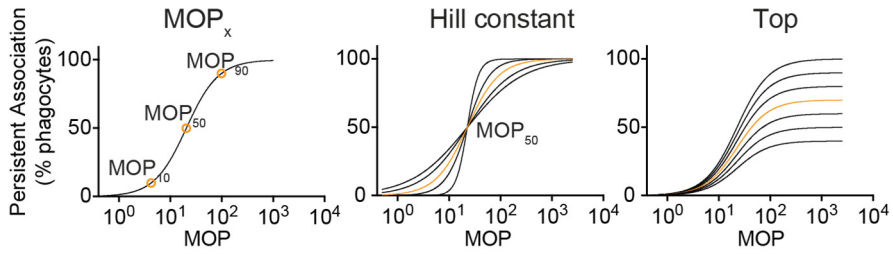

Figure 10

A schematic illustration of different curve characteristics in phagocytosis assessment.

An amendment has been sent to the publisher because in Figure 3A the number shall be in reversed order, the largest volume 1 corresponds to the curve labeled 3 and not 1, as in **Figure 9**.

Key points and findings

- There are several approaches to assessing phagocytosis, but no gold standard, resulting in low reproducibility and comparability in the field.
- Phagocytosis can be described using collision theory and the Hill equation, resulting in a sigmoidal curve with a persistent association as a function of MOP.
- Utilizing PAN enables the normalization of many factors contributing to experimental variation, thereby enhancing the detectable biological signal.
- The curve characteristics can introduce additional information about a system, potentially revealing mechanistic insight.
- For reliable data, it is essential to be within the dynamic range, which can be determined using this approach.

In summary, we established a universal approach to quantify phagocytosis using principles of collision theory and the Hill equation to improve robustness, reproducibility, and sensitivity. This method became the foundation of my thesis and hopefully can be beneficial for others evaluating phagocytosis.

Paper II: A Predictive Model of Antibody Binding in the Presence of IgG-Interacting Bacterial Surface Proteins

Numerous bacteria have evolved strategies to avoid the immune system by altering the binding of antibodies to their surfaces. As previously described, the streptococcal M protein is such a virulence factor that can bind the antibody at the Fc-region thus altering the antibody orientation to a non-immunological binding. This type of binding has been shown for *S. pyogenes* to be more present in niches with low IgG concentrations such as saliva compared to higher IgG concentrations such as serum. This bacterial interference with antibody binding poses a challenge in anticipating the immunological function of antibodies associated with bacteria. To understand the mechanism behind these complex bacterial-antibody interactions a biophysical model was developed, with the focus on describing the interaction between M protein and IgG.

Simplified, an IgG can bind to M protein via either one Fab or the other Fab or the Fc region. These three different binding states compete with one another. In addition, if we add a different monoclonal IgG, it can also bind via the three different states, wherein the Fab binding site may differ. However, if unlucky, the binding site can be completely blocked by the first IgG if they both target the same site or if the sites are close (**Figure 11**). In this case, there is competition for binding to M protein within and in between the two IgG clones and this competition depends on each of the different concentrations and affinities. By adding even more IgGs, it is easy to understand that the situation becomes complex very quickly and needs advanced calculations to model properly, which was done by the first author Vibha Kumra Ahnlide. In this model (**Figure 12**), the M protein is simplified into a linear structure with distinct sites. A polyclonal IgG sample is represented as a range of affinities, and each antibody clone is assigned binding sites on the linear M protein. If the binding site for each IgG clone is not known, the sites are randomly assigned. This is not required for a monoclonal with a known affinity and binding site. For each site, the probability of binding to occur at that site based on the state of the adjacent site can be defined using a transfer matrix, which is an arrangement of elements in rows and columns describing the different binding possibilities. Through calculations, all sites on the M protein are then correlated, allowing us to predict the total binding probability for a given set of parameters.

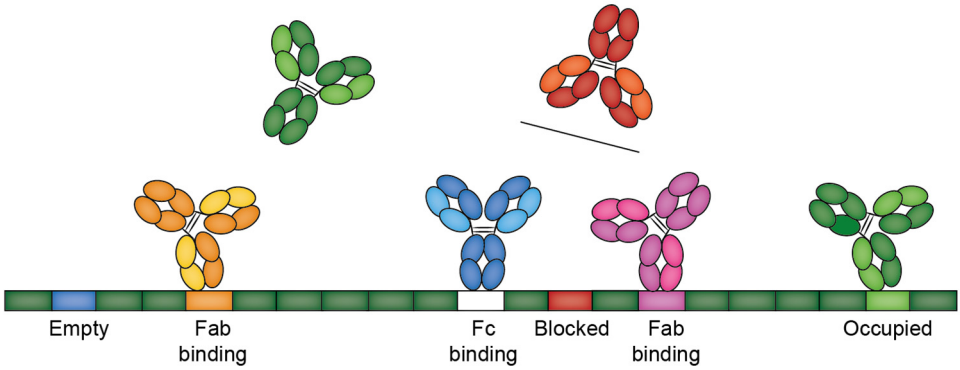

Figure 11

Antibodies can bind to M protein in three different states: via one or the other Fab or via Fc. The binding site can be empty and available for antibody binding or unavailable for binding because it is already occupied by another antibody or is allosterically blocked.

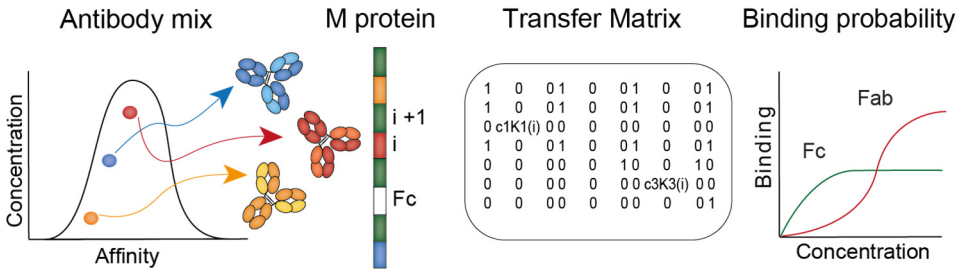

Figure 12

An illustration of the structure of the model. The distribution of antibody affinity is defined and each antibody is assigned a binding site on the M protein. The M protein is modeled as a linear structure with distinctive binding sites. Each antibody is then assessed for the different binding states, where its targeting epitope is "i" and the neighboring site is "i+1". Each state for i and i+1 is described in a transfer matrix with the different binding possibilites expressed as statistical weights. When all antibodies in the mixture have been assessed and their binding correlated, the model can provide a binding probability of how much of the antibodies are binding via Fab respectively Fc depending on the antibody concentration.

This model is unique as it incorporates the number of antibody clones, the concentration of IgG, affinities to Fab and Fc regions, and the location of epitopes on the M protein, which has not been done before. It therefore serves as a valuable tool for investigating the binding interactions between different IgG samples and M protein. The model can with relatively good precision predict if the antibody will bind via Fab or be bound via Fc for various conditions. Using this model to describe the orientation of IgG binding against *S. pyogenes* expressing M protein and M-like protein, the dependence on the IgG concentration, and the affinities for Fab- and Fc-binding were revealed.

Notably, the orientation of binding alone cannot directly be translated into an effector function. However, if it is known that an antibody or a mixture of antibodies is opsonic, we demonstrate that predicted binding via Fab is correlated with phagocytosis. We conducted simulations to examine changes in IgG binding to *S. pyogenes* in serum when specific amounts of known opsonic monoclonal or pooled IgG (intravenous immunoglobulin, IVIG) were introduced and evaluated the local and systemic effects. The monoclonal antibody showed more Fab-binding but saturated at lower concentrations than pooled IgG, resulting in more Fab-binding at high concentrations with pooled IgG. This is probably because pooled IgG contains antibodies that target different binding sites, whereas the monoclonal only has one. Using the approach developed in **Paper I**, phagocytosis experiments established a link between the modified antibody binding and a physiological function, demonstrating the potential for the model to predict the impact of an IgG treatment.

When reviewing this paper for this thesis, a typo in the unit for affinity was discovered and an amendment is planned. The K_D unit is in nM and not nM^{-1} as it is currently presented in this paper, as well as **Paper IV**. No conclusions are affected.

Key points and findings

- Competitive antibody binding is dependent on affinities, binding geometry, clonality, concentration, and epitope localization.
- A method for modeling competitive IgG binding against IgG-interacting bacterial surface proteins is developed using the transfer matrix model.
- This model can describe and predict binding for both polyclonal and monoclonal antibodies against M protein in a physiological context with varying affinities, concentrations, epitopes, and localization.
- The predicted binding for known opsonic antibodies correlates well with phagocytosis.

This study gives a mechanistic understanding of bacterial antibody targeting and provides a tool for predicting the effect of antibody treatments in the presence of bacteria with IgG-modulating surface proteins. My main contribution as a co-author was in the assessment of phagocytosis. This model was later used in **Paper IV** to describe the antibody response during invasive *S. pyogenes* infection.

Paper III: Lack of Opsonic Antibody Responses to Invasive Infections with *Streptococcus dysgalactiae*

S. dysgalactiae can cause recurrent invasive infections in contrast to *S. pyogenes*. This difference is somewhat surprising as they otherwise have similar disease manifestations and share many of the virulence factors, described in more detail in Chapter 4. Considering the limited knowledge about *S. dysgalactiae* immunity, and that the immunity to *S. pyogenes* has long been primarily attributed to a type-specific antibody response, we sought to explore the antibody response during *S. dysgalactiae* bacteremia in **Paper III**. Patients with *S. dysgalactiae* bacteremia in the county of Skåne between 2017 and 2018 were prospectively included after informed and written consent. Sixteen patients were included by the first author Anna Bläckberg. The most common infection site was soft tissue and all patients had comorbidities with the most prevalent being heart failure and previous malignancies, followed by diabetes mellitus and lymphoedema. The youngest was 55 and the oldest 86 years old, the median being 80. During the study period, a single patient was identified with recurrent bacteremia caused by *S. dysgalactiae*. While there may have been more patients with recurrent bacteremia, the duration of the study was limited.

Acute sera were collected within 5 days of hospital admission, while convalescent sera after 4–6 weeks. The sera was analyzed for the development of type-specific antibodies, and the infecting isolates were *emm* typed. In almost all patients, the total levels of IgG increased after infection, and in several cases, the development of IgG antibodies against the infecting isolate was detected. Type-specific antibodies were demonstrated only in the convalescent serum in the patient experiencing a recurrent infection against the recombinant M protein that matched the infecting *emm* type.

To further evaluate these type-specific antibodies, the Lancefield bactericidal assay, which was described in Chapter 2, was employed to quantify the phagocytic killing mediated by the sera. The convalescent sera containing the type-specific antibodies did not improve bacterial killing, if anything, the contrary was observed. These assays do not measure phagocytosis directly and can be difficult to perform and reproduce. Therefore, to complement the bactericidal assay, the opsonic capacity of the three sera was evaluated using the PAN-method from **Paper I**. During and after recurring infection, both association and internalization were slightly improved on a phagocyte population level. On an individual phagocyte level, however, not statistically significant, the tendency to a decrease in phagocytic ability was seen for both adhesion and internalization of the infecting isolate. Together, this exemplifies how phagocytosis assessment on both population and individual phagocyte levels can be important to perform, but also how different phagocytosis methods can complement each other.

The opsonic capacity of the serum containing type-specific antibodies was complex and not very clear. Therefore the lack of enhanced bactericidal effect seen by that serum might partly be explained by the development of non-opsonic type-specific antibodies, in other words, antibodies not facilitating phagocytosis. Nevertheless, the results from the bactericidal assay can of course have other explanations as well.

Key points and findings

- Type-specific antibodies can be developed after an invasive *S. dysgalactiae* infection.
- These antibodies were developed after a recurrent infection and did not mediate bacterial killing, nor were clearly opsonic.
- Phagocytosis showed a slight overall improvement during and after recurrent infection, but at the individual phagocyte level, both adhesion and internalization exhibited a tendency to decrease.
- The lack of opsonic antibodies after infection may partially explain why invasive infection with *S. dysgalactiae* recurs.

In conclusion, *S. dysgalactiae* bacteremia may lead to increased antibody levels including type-specific ones, but the study did not find evidence that these type-specific antibodies were neither effectively bactericidal nor opsonizing. The failure to produce functional antibodies might partly explain why *S. dysgalactiae* can cause recurrent invasive infections in the same host. In this paper, my primary contribution as a co-author involved quantifying the opsonic capacity with the direct assessments of phagocytosis.

Paper IV: Invasive Streptococcal Infections Can Lead to the Generation of Cross-Strain Opsonic Antibodies

The interaction between *S. pyogenes* and our immune defense has long been a subject of research interest, particularly in pursuit of understanding how immunity is developed, and how an *S. pyogenes* infection can be prevented or at least cleared. In Chapter 4 the current understanding of this is presented, but our knowledge about long-term protection against *S. pyogenes* is still insufficient to protect millions of people each year from suffering. Therefore, further research is needed to battle this dangerous pathogen. In this study, we meticulously examined the opsonic antibody response following invasive *S. pyogenes* infection in four patients with bacteremia between 2018 and 2020 at Skåne University Hospital in Lund, Sweden.

Similar to **Paper III**, Anna Bläckberg, here as co-author, enrolled the patients and collected the acute- and convalescent-phase sera. The infecting *S. pyogenes* isolates were genome-sequenced with the results *emm*118, *emm*85, and two *emm*1 isolates. In general, the patients were previously healthy with no severe comorbidities, and a median age of 60, the youngest being 34 and the oldest 73. Skin and soft tissue were the primary infection foci. The acute-phase serum from the patient infected with isolate *emm*85 might have been in a different immune response phase than the other acute-phase samples because his symptoms commenced two weeks before being admitted to the hospital, and in addition developed septic shock.

The total IgG concentration was increased after the infections for all patients, with some differences in isotype distribution. Antibody binding was assessed against the infecting isolate experimentally and then described using the model from **Paper II**. All patients exhibited increased antibody binding after the infection against its infecting isolate, but also across the other infecting isolates. The opsonic capacity of this antibody binding was later evaluated on its ability to mediate phagocytosis against the corresponding isolate. Through meticulous quantitative phagocytosis assessment using the approach developed in **Paper I**, we could determine a differential opsonic response after invasive *S. pyogenes* infection. When summarizing the analyzed parameters, one patient exhibited a clear enhancement in phagocytosis at both the population and individual phagocyte levels, while another

patient showed some improvement. The other two had no improvement, but one of them, the patient with septic shock, consistently demonstrated the highest overall opsonic ability already in the acute-phase serum, which persisted in the convalescent-phase serum.

To ascertain if our observations were specific to the *emm* type we evaluated the opsonic capacity across different isolates. In summary, the data indicates an increase in phagocytosis in all convalescent-phase sera, albeit not for all isolates but at least one per patient. Hence, following invasive *S. pyogenes* infection, cross-strain opsonic antibodies may be produced. Conversely, non-opsonic binding antibodies can be generated against specific types, in this present work particularly observed with the *emm*1 type. The clinical outcome could not clearly be linked to any of the results in this limited study population.

The amendment for **Paper II** also affects this paper, nM is the correct unit for K_D, but no conclusions are affected.

Key points and findings

- Antibody binding is increased after invasive *S. pyogenes* infection against the infecting isolate but also across strains.
- Invasive *S. pyogenes* infections lead to differential opsonic response, some mediates improved phagocytosis while others do not.
- Infection-induced opsonic antibodies after invasive *S. pyogenes* infection are cross-reactive, while the non-opsonic response seems to be *emm* type specific.
- Antibody binding is not equivalent to opsonic response in the context of *S. pyogenes* infection.

In conclusion, invasive *S. pyogenes* infections result in modestly increased antibody binding with varying opsonic capacity, encompassing both non-opsonic and broadly opsonic binding across types. These findings challenge the prevailing notion that invasive *S. pyogenes* infections should lead to a strong type-specific antibody response rather than a more modest but broadly reactive response. Additionally, our results suggest that an increase in antibody titers may not always indicate an opsonic response, emphasizing the need for a nuanced understanding of antibody function in the context of *S. pyogenes* infections. The study contributes valuable insights to our overall understanding of how immunity to invasive *S. pyogenes* infection can develop by highlighting the emergence of cross-opsonic antibodies, which may be important for long-term immunity against this pathogen.

I consider this paper a fitting ending to my Ph.D. journey. The research question originated from the productive collaboration in **Paper III**, which seamlessly

extended into this project. Moreover, a substantial part of the experimental work was carried out by two students, Hanna Ivarsson and Sofia Thomasson, under my supervision, contributing to my growth as a scientist and as a person. Ultimately, this project brings together the methods from **Paper I** and **Paper II**, placing them in a more clinically relevant context. As the culmination of my doctorate, this paper weaves together the diverse experiences from my previous work, and has deepened my understanding of each one of them.

Discussion

"The only true wisdom is in knowing you know nothing."

Socrates

In science we all aspire to understand and reveal the truth, however, the truth in our hands is uncertain, and will always be. This can be daunting, but for me, it is humbling and encouraging. I have come to the belief that a good scientist not only needs to learn how to accept but also how to embrace the uncertainty of the truth as well as the unknown. In light of this, I will now proceed to discuss the overarching themes and key insights of this thesis, focusing on their potential contributions to the field.

The role of dose-response curves in assessing phagocytosis is a central theme of this thesis. Initially labeled as PAN, the approach aimed for assessments at MOP_{50}. However, over the years and through different projects our understanding has evolved. In scenarios of low experimental variation, examining the full curve offers richer insights than reducing it to one data point. Conversely, it is still advantageous for discerning the phagocyte population effect from individual phagocyte assessments, in systems with high experimental variation, or for standardized measurements across systems.

The methodology, or its various adaptations, has played a pivotal role in numerous projects within our lab and has started to extend its reach to other research groups that explore phagocytosis and antibody functions.[127,140,278–280] While this thesis concentrates specifically on evaluating antibody-mediated phagocytosis, it is noteworthy that others have successfully applied this method to study complement-mediated as well.[280] Its value lies in its simplicity and robustness between users, making it user-friendly and more consistent compared to other approaches. The method's adaptability to different prey, opsonins, and cell types further amplifies its versatility.

However, despite its application to diverse systems, the method remains confined to a specific facet of this intricate process. Within this thesis, phagocytosis has primarily been studied using a single cell line together with fluorescently labeled heat-killed bacteria, and the data acquired through flow cytometry. This approach

represents a streamlined system, emphasizing the initial phases of phagocytosis from the perspective of FcγR-IgG-antigen interaction. As detailed in the methodological considerations, this simplified study design is both a strength and a limitation. The study design and approach employed in this thesis complement other methods for studying phagocytosis. While we quantify the initial interaction, subsequent steps like phagosome maturation and killing are not captured. To gain a comprehensive understanding of phagocytosis, different methods are necessary. Our approach can be used as a high-throughput, quantifiable screening tool, such as evaluating the opsonic capacity of antibodies, to discover conditions that are valuable to study in detail with additional methods.

It is essential to emphasize that PAN is not merely a phagocytosis assay but rather an approach designed to systematically evaluate phagocytosis. Its versatility extends to various applications, because the association of phagocytes, delineated as a function of MOP, relies on principles independent of study design. Nonetheless, adapting the Hill equation may be necessary for a more accurate fit in complex systems with multiphasic features. One potential approach, as described by Giovanni Veroili in pharmacology studies, views each phase of the curve as an independent dose-dependent process [165].

Others have developed flow cytometric-based OPAs as a high-throughput alternative to the more traditional killing OPAs, described in Chapter 2. For *S. pneumonia* and group B streptococcus, internalization OPA and killing OPA correlate well[281,282], however, killing OPAs have been more common in evaluating vaccine-induced protection for *S. pneumonia*.[93] For both types of OPA a serum dilution is assessed with the titer dilution resulting in 50 % internalization or killing is reported. Through optimizations, one MOP value could be chosen reducing the number of samples to assess.[83,281,282] These approaches share many of the concepts we present in **Paper I**, which strengthen its foundation and exemplify its advantage of cross-laboratory comparisons. However, as per my understanding, the resulting curve is not further evaluated, and MOP-curves are not utilized to assess the system's dynamic range or to reduce variability when necessary. Therefore, by making only minor adjustments to existing methods, this thesis contributes to a more systematic approach to quantifying phagocytosis, offering the advantage of additional parameters and yielding more robust data. Even though not evaluated in this thesis, killing and other phagocytic effector functions that are dependent on MOP can probably be assessed by a similar approach. Hopefully, this thesis can contribute not only to the methodology of phagocytosis assessment, but also to a broader perspective on data assessments and inspire others in this regard.

While the primary focus of this thesis has been on accurately quantifying phagocytosis, for me its significance lies in placing this understanding within a broader, more clinical context such as host-pathogen interactions. For a long time,

the presence of specific antibodies was believed to be sufficient to confer immunity, and for most available vaccines titers are used as surrogates for evaluating protection.[283] However, it has been shown that specific titers do not always guarantee immunity and correlates can vary depending on the type of protection, pathogen, and individual characteristics.[127,283,284] Instead, it has been proposed to evaluate the antibody response based on whether it is functional or non-functional. The term non-functional refers to antibodies that bind to their antigen without actively contributing to the neutralization or eradication of the pathogen. Consequently, a non-opsonic antibody binds but does not facilitate phagocytosis.[48]

The mechanisms that induce the production of non-functional antibodies, rather than functional ones, are thought to be advantageous for the pathogen and its virulence. This phenomenon has been recently elucidated by Uddén et al for *S. pneumonia*, where the generation of a non-opsonic antibody response was correlated with the invasiveness of the infection.[285] It is not far-fetched to suspect that other pathogenic streptococci also have evolved similar strategies. In this thesis, we found such an example with non-opsonic antibodies as a potential explanation for recurrent *S. dysgalactiae* invasive infection, something that had not been described previously.

Furthermore, the antibody responses we detected after invasive *S. pyogenes* infections were diverse with the development of both non-opsonic and cross-strain opsonic antibodies. Noteworthy, the same type (*emm*1*)* resulted in two distinctive responses, emphasizing how intricate the interaction between the pathogen and the host is during invasive *S. pyogenes* infections. While these responses do not directly translate to immunity, they shed light on potential mechanisms for protection. Our findings strengthen the rising notion that not only type-specific antibodies but also cross-opsonic can be relevant for *S. pyogenes* immunity[266–268,286]. Even if this study is small, this type of in-depth assessment of clinically invasive *S. pyogenes* infection is unique and therefore can contribute to the general understanding of how immunity against *S. pyogenes* may be developed.

Moreover, this thesis demonstrates how functional assessments and mathematical modeling can enhance our understanding of antibody responses. This approach has subsequently been extended to other projects, as exemplified in a collaboration with Wael Bahnan. In that project, a cross-opsonic anti-M antibody was discovered and evaluated after a non-invasive *S. pyogenes* infection, presenting potential clinical value[269]. This antibody was assessed in **Paper II** and compared against the currently available therapeutic IVIG. The high-affinity monoclonal was slightly more opsonic at low concentrations compared to IVIG but was out-concurred by the multivalency of IVIG at higher concentrations. These findings suggest the potential benefit of combining their traits through a cocktail of high-affinity multivalent antibodies. An

insight that not only might be relevant for therapeutic purposes but may also hold significance for vaccine development.

S. pyogenes is not unique in its intricate interaction with our immune system. In general, the interaction between host and pathogen is a complex process that ranges from symbiosis to life-threatening diseases. Over years of evolution, the immune system has evolved numerous strategies to clear infections and protect the host from disease. Insights from these strategies have been instrumental in the development of effective therapeutics and vaccines against other pathogens. At present, there is no approved vaccine against *S. pyogenes,* but many of the currently available vaccines against other pathogens are thought to provide protection via antigen-specific antibodies.[283] However, antibodies can confer protection through multiple mechanisms, and often the final result depends on a collective combination of different responses in a dynamic equilibrium within the immune environment provided. To approach this complexity, inspired by Systems Biology, Galit Alter with colleagues introduced Systems Serology. It consists of a combination of high-throughput experimental techniques aimed at characterizing antibody features and quantifying antibody functions. Computational methods are then used to provide a deeper understanding of the humoral immune responses.[287] Throughout this thesis a similar approach has been employed. First, high-throughput functional assay and computational modeling were developed in **Paper I** and **Paper II**, and then these were applied to clinical questions in **Paper III** and **Paper IV**. Together each paper in this thesis systematically contributes to a better understanding of the antibody response in streptococcal infections.

Although this thesis belongs within the basic research field, it aspires to encompass clinical aspects as well, demonstrating the advantages of translational multidisciplinary work. Hopefully, the insights gained, and more importantly, the approach adopted throughout my Ph.D. studies, will contribute to the work of others. In our general quest to understand the truth of the world a little bit better I hope this thesis enhances the understanding of the world of phagocytosis and antibody responses in invasive streptococcal infections.

Concluding remarks

"This too shall pass."

In conclusion, this thesis aimed to develop a universal and robust method for quantifying phagocytosis and assessing antibody response in streptococcal infections. The novel approach based on the principles of the Hill equation is versatile, quantifiable, and robust, resulting in improved quality of phagocytosis assessment. It provides valuable insights into the opsonic capacity of antibodies, particularly appreciated in this thesis when studying the antibody response during invasive *S. pyogenes* and *S. dysgalactiae* infections. The diversity observed highlights the importance of quantifying antibody function in understanding this critical host-pathogen interaction. In conclusion, this thesis contributes to the overall understanding of how to quantify antibody-mediated phagocytosis in the context of invasive streptococcal infections.

Future perspectives

"Someone is sitting in the shade today because someone planted a tree a long time ago."

Warren Buffet

An obvious continuation of this thesis is to delve deeper into understanding the different curve characteristics, aiming to uncover mechanistic explanations with the hope of using this knowledge to predict and explain different types of opsonic antibody responses in the future. Furthermore, we can continue with an envisioned application that would incorporate such data into a comprehensive database on phagocytosis characteristics across diverse conditions. This database would function as a library on various phagocytes and prey under different opsonizing or clinical conditions. Systems with limited, sensitive data, including diverse clinical isolates and patient material, would benefit from the generation of high-quality data that could be archived in the database, thereby making it accessible over time. Similar to other fields, such a repository would probably be valuable for mining data, facilitating the identification of potentially significant preclinical and clinical aspects of phagocytosis.

Another aspect revolves around the potential implication this thesis might have on the broader scientific community. It is my deepest hope that others who assess phagocytosis and the opsonic capacity of antibodies can be inspired and find the approaches developed in this thesis useful. Through certain adjustments, these approaches might find practical implementation in clinical settings where antibody function can be of relevance, or in vaccine studies as correlates for protection or when screening for potential therapeutics with opsonic capacity. If one is allowed to expand the horizon even further, I believe that these are implications not only for infection medicine but for various fields where phagocytosis and antibodies are involved.

However, the future prospects for research on *S. pyogenes* and *S. dysgalactiae* appear endless. It is daunting trying to grasp the extent of what still needs to be unraveled to comprehensively understand these specific host-pathogen interactions. Yet, it is a challenge not without hope. Ever since Hippocrates described their horrible diseases, a tireless quest has been undertaken by the whole community, and

I believe it will persist as long as necessary. Building upon the insights from this thesis, a natural continuum is further assessing the diversity in opsonic responses following streptococcal infection. Exploring these responses in distinct populations and considering different levels of infection severity could be a prospect for future studies.

As I approach the completion of this thesis, I do not see an ending but rather a new beginning. The future can, and hopefully will, behold more than I possibly can imagine, and I am curious to discover what tomorrow might bring.

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"It takes a village to raise a child."

I asked fellow researchers for advice on how to write my thesis, and many told me to start with the most important section. Consequently, I started with acknowledgments. Some of the people I have had the honor to meet thanks to this research have influenced me more than I can possibly express. They have inspired me and taught me, not only about science, but given me valuable lessons for life. I am grateful for all of you.

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Afterword

I believe it is unusual to have an afterword in a doctoral thesis; it is certainly not necessary, but I am not ready to finish this journey without acknowledging the impact it has had on me as a person.

This doctorate has been about more than learning how to conduct research; it has extended beyond learning laboratory work, planning projects, writing manuscripts, applying for grants, and answering scientific questions. For me, it has been a true challenge that has changed my perspectives and way of thinking. The quotes included in this thesis collectively symbolize this change, and each one holds personal significance to me.

I learned that science is not easy. You probably cannot imagine how many times I, or at least my experiments, have failed over the years. Rising after a fall, fundamental in judo, is a lesson for which I have been very grateful during this journey. It can be tough, but possible and, at times, inevitable. However, the real challenge for me was learning to embrace the possibility that, despite my best efforts, it might not work out. This is the nature of research. To be aware of this and still persevere is a testament to grit, passion, or sometimes, for lack of a better term, a venture close to insanity. Thanks to the people around me, I continued, fueled sometimes by passion and sometimes by grit, striving to stay as grounded as possible. I learned I had to change my perspective and understand that as long as I worked diligently and did my best, there was value in the effort.

Furthermore, as a student, you are often taught in school that there are right and wrong answers. Sometimes the key can be wrong, but then there is another answer that is right. In science, you will never have an answer like that. We strive to answer questions and aim for the truth, but we will never know for sure. This realization was at first unsettling and somewhat provoking, but in the end, after changing my perspective, it became exciting and humbling. There is no perfect answer, no perfect method, and no perfect thesis.

But there is curiosity, and there is a whole world out there ready to be explored.

Enjoy!

Therese de Neergaard,

The author

Lomma, 18th of January, 2024

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