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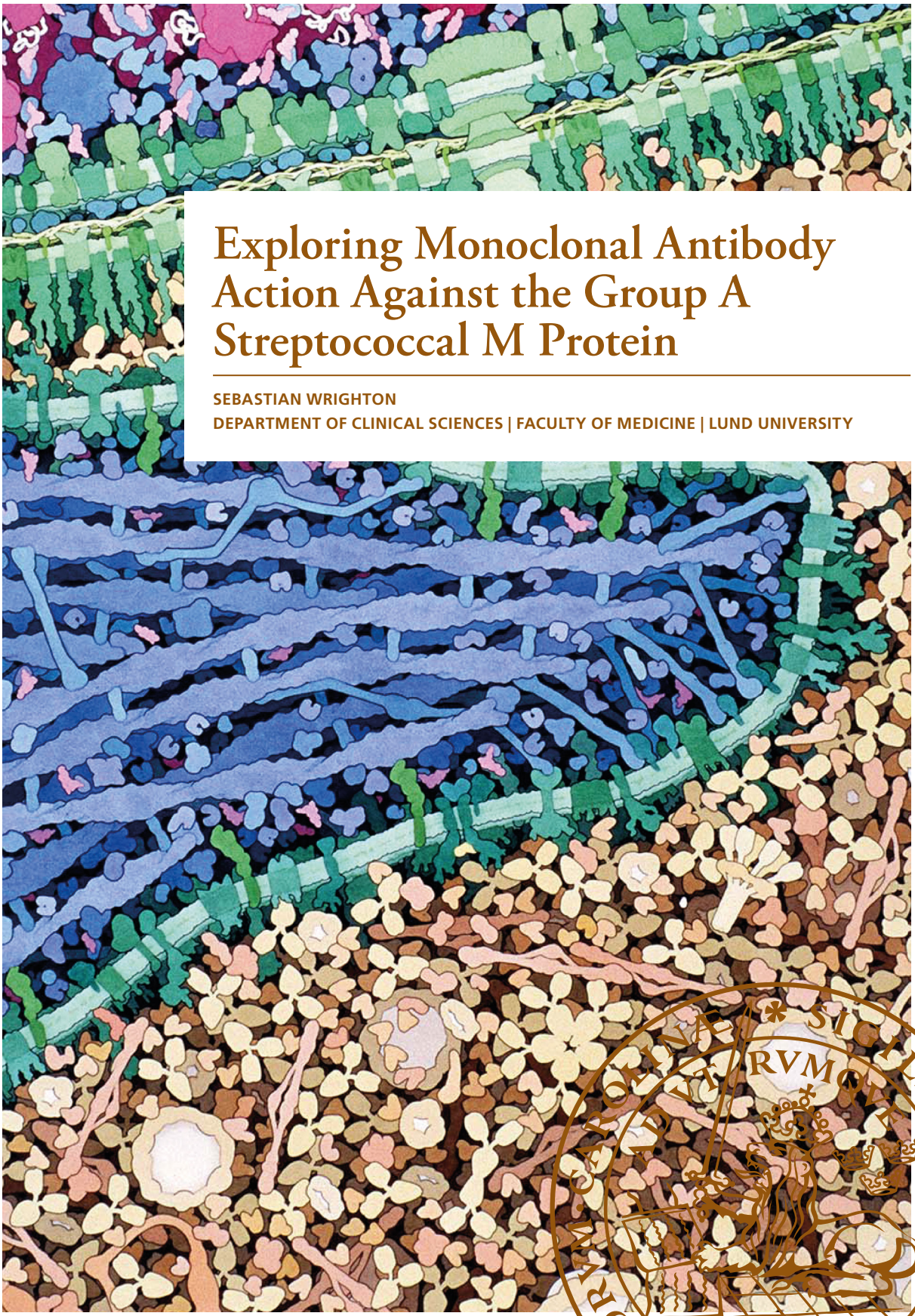

Exploring Monoclonal Antibody Action Against the Group A Streptococcal M Protein

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

With due permission from the Faculty of Medicine, Lund University, this doctoral thesis will be publicly defended on the 13th of October 2023 at 13:00 in Segerfalksalen, Biomedical Center (BMC), Lund, Sweden.

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Summary: Group A Streptococcus (GAS) is a significant human pathogen that has developed multiple immune evasion mechanisms to counter the host immune response. One of these mechanisms involves the production of the M protein, which, amongst other things, acts as an anti-phagocytic factor and can bind host proteins. Another is the ability of M protein to bind a human protein called fibronectin (Fn). This protein plays a key role in a number of physiological processes and can be used by GAS to evade the immune system. In this PhD dissertation, we aimed to assess the binding efficacy and function of monoclonal antibodies targeting the GAS M protein.

In the first paper we start by developing a robust method to assess phagocytosis. This method highlights the importance of factors such as volume, time, and the ratio of phagocyte to prey on the phagocytic process. It has allowed us to, henceforth, attain precise, high quality phagocytosis data and has been a major driving force for other projects within the lab – especially the three other papers included in this thesis.

In the second paper we discovered a novel form of antibody binding whereby a monoclonal binds the GAS M protein in a bivalent dual-Fab cis mode. This means that both Fab arms of the Ab bind to distinct epitopes on the target molecule simultaneously. Even so this antibody bound to a region of the M protein associated with non-opsonic antibodies we found that this Ab could enhance phagocytosis suggesting that this novel binding form can circumvent the M protein's anti-phagocytic properties.

In the third paper we investigated the M protein's ability to bind fibronectin. While this function was described in previous studies, we found it could only do so with very low affinity. We found that the binding of antibodies from the blood of donors who had recently recovered from a severe GAS infection could greatly enhance this fibronectin binding. We show that same occurs with certain anti-M monoclonals and that this mechanism leads to a reduction in opsonophagocytosis. Moreover we find that Ab flexibility may play a role and that Ab Fc domains are a crucial factor in mechanism.

In the fourth paper we further explore this anti-phagocytic effect. Here we assess the effects of varying concentrations of Fn since this can differ greatly within the human body. We found that both very low and high concentrations of Fn, corresponding with the nasopharyngeal niche and blood respectively, led to a substantial reduction in phagocytosis. We moreover found that this reduction in phagocytosis is likely linked to a modulation of integrins. Overall, this work provides insights into immune evasion mechanisms developed by GAS and highlights how this remarkable pathogen always seems to be one step ahead of us.

Key words: group A streptococcus, *Streptococcus pyogenes*, fibronectin, phagocytosis, innate immunity, antibodies, adaptive immunity

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Coverart (front and back): This is a cropped version of the painting titled ‘Macrophage and Bacterium 2,000,000X’ and was painted by the artist and scientist David Goodsell. The illustratrion shows a cross section of a macrophage in the process of engulfing a bacterium. It gives a detailed perspective of the point of interaction between the two cells. It meticulously highlights the individual macromolecules that play a role in the process of immune recognition, as well as those that are essential for the basic processes of life. The painting is used here with the artist’s permission.

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Standing on the shoulders of giants

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Popular Scientific Summary

Group A Streptococcus, commonly known as GAS, is a devious bacterium that often infects humans. It usually causes relatively mild conditions like ‘strep throat’ but can rarely cause very severe illness which is why they are sometimes referred to as ‘flesh eating bacteria’. This bacterium has evolved many techniques to avoid our immune system. One of its most cunning tricks is making a protein called the M protein. This protein not only protects the bacterium from being eaten by our immune cells but can also stick to our own proteins to help it to hide from immune defenses. One protein it clings to is called fibronectin and it is vital for many of our body's processes. The goal of this PhD thesis was to better understand how antibodies, special proteins made by our immune system, interact with the M protein and how this can affect the M proteins ability to stick to fibronectin.

In the first paper we established a reliable way to measure how immune cells protect us by ‘eating’ invaders like GAS bacteria, a process called phagocytosis. This method let us account for factors like reaction volume, incubation time, and the number of bacteria per immune cell since these can influence how efficiently they eat. With this tool in hand, we were able to get clearer data that boosted other projects in our lab, including the other papers included in this thesis.

In the second paper we found a new and interesting way that certain antibodies stick to the M protein. Imagine an antibody as a 'Y' shape; in this case both arms of the 'Y' can attach to different spots on the M protein at the same time. This is a new finding because usually antibodies only bind to a target once. Even though these antibodies stick to a part of the M protein that usually helps GAS evade our defences, they surprisingly make it easier for our immune cells to eat GAS.

In the third paper we looked closely at how the M protein binds to fibronectin. While previous studies noted this behavior, we observed that the M protein doesn't stick very tightly on its own. But when antibodies from people who had recently recovered from a tough GAS infection were present, the M protein's grip on fibronectin got much stronger. This tighter bond makes it harder for our immune cells to eat and remove GAS from our bodies.

Finally in the fourth paper, we studied how different amounts of fibronectin influence GAS's protection from being eaten by immune cells. We discovered that both very low and very high levels, found in different parts of our body, make it hard for our immune cells to eat GAS. We saw that this protective effect might be because of changes in our body's proteins known as integrins. These help cells communicate, attach to their environment, and are even involved in the process of ‘cell eating’.

Populärvetenskaplig sammanfattning

Grupp A-streptokocker, allmänt kända som GAS, är en lömsk bakterie som ofta infekterar människor. Den orsakar vanligtvis relativt lindriga tillstånd som "halsfluss" men kan i sällsynta fall orsaka mycket allvarliga sjukdomar, vilket är anledningen till att de ibland kallas "köttätande bakterier". Denna bakterie har utvecklat många sätt för att undvika vårt immunförsvar. Ett av dess listigaste knep är att tillverka ett protein som kallas M-protein. Detta protein skyddar inte bara bakterien från att bli uppäten av våra immunceller, utan kan också fästa till våra egna proteiner för att hjälpa den att gömma sig från immunförsvaret. Ett protein som den fäster vid kallas fibronektin och det är avgörande för många av kroppens processer. Målet med denna doktorsavhandling var att bättre förstå hur antikroppar, speciella proteiner som tillverkas av vårt immunsystem, interagerar med M-proteinet och hur detta kan påverka M-proteinets förmåga att fästa till fibronektin.

I den första artikeln utvecklade vi ett sätt att mäta hur immunceller skyddar oss genom att "äta" inkräktare som GAS-bakterier, en process som kallas fagocytos. Med den här metoden kunde vi ta hänsyn till faktorer som reaktionsvolym, inkubationstid och antalet bakterier per immuncell, eftersom dessa kan påverka hur effektivt de äter. Med det här verktyget i handen kunde vi få tydligare data som gynnade andra projekt i vårt labb, inklusive de andra artiklarna som ingår i den här avhandlingen.

I den andra artikeln fann vi ett nytt och intressant sätt för vissa antikroppar att fästa vid M-proteinet. Föreställ dig en antikropp som ett Y. I det här fallet kan Y:ets båda armar fästa på olika ställen på M-proteinet samtidigt. Detta är en ny upptäckt eftersom antikroppar vanligtvis bara binder till ett mål en gång. Även om dessa antikroppar fäster vid en del av M-proteinet som vanligtvis hjälper GAS att undvika vårt försvar, gör de det överraskande nog lättare för våra immunceller att äta upp GAS.

I den tredje artikeln tittade vi närmare på hur M-proteinet binder till fibronektin. Tidigare studier har visat att M-proteinet binder till fibronektin, men vi kunde konstatera att det inte gör det på egen hand. Men när antikroppar från personer som nyligen hade återhämtat sig från en tuff GAS-infektion var närvarande, blev M-proteinets grepp om fibronektin mycket starkare. Denna tätare bindning gör det svårare för våra immunceller att äta och avlägsna GAS från våra kroppar.

I den fjärde artikeln studerade vi hur olika mängder fibronektin påverkar GAS skydd mot att ätas upp av vara immunceller. Vi upptäckte att både mycket låga och mycket höga nivåer, som finns i olika delar av vår kropp, gör det svårt för våra immunceller att äta GAS. Vi såg att denna skyddande effekt kan bero på förändringar i kroppens proteiner som kallas integriner. Dessa hjälper celler att kommunicera, fästa vid sin omgivning och är till och med inblandade i processen för "cellätande".

Abbreviations

ADCC	Ab-dependent cell-mediated cytotoxicity
Ab	Antibody
AMP	Antimicrobial peptide
APC	Antigen presenting cell
CD	Cluster of differentiation
CFU	Colony forming unit
CH	Constant heavy (domain)
CR	Complement receptor
CLR	C-type lectin receptor
DFCB	Dual-Fab cis-binding
ECM	Extracellular matrix
EC_{50}	Half-maximal effective concentration
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcR	Fc receptor
FcγR	Fc gamma receptor
Fn	Fibronectin
FnBP	Fibronectin-binding protein
GAG	Glycosaminoglycans
GAS	Group A streptococcus
GPIIb/IIIa	Glycoprotein IIb/IIIa
HEK-293	Human embryonic kidney 293 cells
HVR	Hypervariable region
IgG	Immunoglobulin G
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex

NADase	NAD^+ glycohydrolase
NCAM	Neural cell adhesion molecule
NK cells	Natural killer cells
RHD	Rheumatic heart disease
MAMP	Microbe-associated molecular pattern
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
SAg	Superantigen
STSS	Streptococcal toxic shock syndrome
TCR	T cell receptor
VDJ	Variability, diversity, and joining
VF	Virulence factor
Vn	Vitronectin
VR	Variable Region

1. Systems of Immunity

The Dynamic Duo: A Short Introduction to Innate and Adaptive Immunity

The human immune system is a complex network of cells, tissues, and organs that operates as a highly effective guardian of our health. It is tasked with the critical mission of protecting the body from disease. This could be triggered by pathogens like bacteria, viruses, fungi, and parasites or noxious environmental toxins and potentially cancerous cell mutations. The immune system works tirelessly, coordinating a response to threats in real-time to maintain our wellbeing. Fundamentally, the immune system is broadly categorized into two primary branches: the innate immune system and the adaptive immune system. Both these systems interact and cooperate in the shared goal of protecting our body from disease, but each has its unique mechanisms and responsibilities.

The innate immune system, often referred to as our body's first line of defense, offers broad, rapid protection against pathogens (1). This component of our immune system includes physical barriers such as our skin and mucous membranes that physically prevent the entry of threats (2). Further, there are chemical barriers such as gastric acid or bile acid and biological barriers such as digestive enzymes, mucus, and saliva (3). Finally, there are the cells of the innate immune system which comprise certain white blood cells (also known as leukocytes) or a plethora of other, heterogenous cell types (4,5). Innate immunity is quick to respond, offering immediate defense against infection. This is, in part, possible due to mechanisms such as inflammation, which helps in localizing the infection. Inflammation is triggered by molecules that are discharged by cells that have sustained damage or have sensed a threat through other means. It essentially creates a protective barrier to prevent the further spread of an infection and facilitates the repair of any damaged tissue once the pathogen or threat has been eliminated (6). Further, the complement system is a biochemical cascade that assists, or ‘complements’, the capacity of antibodies (Abs) to eliminate pathogens or signal them for removal by other cells (7).

The innate immune system is, often referred to as the ‘non-specific’ arm of the immune system. This is somewhat misleading since the cells of the innate immune system can act with high precision. They do so by recognizing and reacting to

conserved signals and patterns associated with harmful pathogens and other dangerous substances (8). The critical difference lies in the fact that they cannot adapt and recognize novel foreign intruders since their specificity is inherited in their genome (1). Equipped with defense mechanisms like phagocytosis and cytotoxic molecules, these cells can swiftly initiate a protective response (4). A range of distinct immune cells are included in the innate immune system. Professional phagocytes, such as macrophages, B cells, and dendritic cells, protect the body by ingesting and so removing pathogens and foreign particles from the body. Then there are granulocytes such as basophils, eosinophils, and mast cells. These cells derive their name from the granules they hold in their cytoplasm. These granules are secretory vesicles that store a mixture of cytotoxic molecules, including many enzymes and antimicrobial peptides (AMP). Neutrophils constitute an interesting cell type since they are both professional phagocytes and granulocytes (1). Even cells that we would usually not associate with the immune system like epithelial cells or mesenchymal cells are a part of the innate immune system since they serve as non-professional phagocytes. They do so by, for example, ingesting neighboring apoptotic tumor cells (9).

While the innate immune system offers a rapid response, it lacks specificity and memory. It responds to invaders in essentially the same way each time, without the ability to remember and recognize specific pathogens in the future. This is where the adaptive immune system steps in. However, we are not yet done with the innate immune system since it plays a crucial role in the stimulation and eventual activation of the adaptive immune system (10). Some professional phagocytes are also professional antigen-presenting cells (APC). These phagocytes express heterodimeric molecules known as the major histocompatibility complex (MHC) class II. When an APC ingests foreign, or nonself proteins, these are eventually fragmented through digestive enzymes. The resulting short chains of amino acids known as peptides are loaded onto the MHC class II molecule which is then expressed on the cell surface. This MHC class II-peptide antigen complex can then interact with the T cell receptor (TCR) of cluster of differentiation (CD) 4^+ T cells (also known as T helper cells). In turn these $CD4^+$ T cells can, if necessary, initiate an adaptive immune response against the foreign entity (11). As mentioned above, a wide range of cell types can function as non-professional phagocytes. These cells, along with virtually every other type of human cell, also serve as non-professional APCs. They do so through expression of the MHC class I molecule. In contrast with MHC class II, this molecule is primarily used to present peptides derived from endogenous proteins (also known as self-proteins). This means its main function is to allow the immune system to assess whether cells are healthy and acting normally. This occurs through patrolling $CD8^+$ T cells (also known as cytotoxic T cells) that use their TCR to assess the self-peptides loaded onto the MHC class I molecule. This is particularly important for cancer prevention since essentially any cell can succumb to mutations that allow for its uncontrolled proliferation and malignant

transformation (12). Moreover, this pathway is crucial for the identification and eradication of cells infected with viruses (13).

The adaptive immune system is a more specialized and targeted form of defense. Unlike the innate system, adaptive immunity is capable of remembering specific pathogens, allowing for a quicker and more potent response upon subsequent encounters. This characteristic is why vaccines work – they introduce a harmless form of the pathogen into the body, allowing the adaptive immune system to, in the case of a true exposure, recognize the invader and respond more efficiently (14). The adaptive immune system's key players are the T cells and B cells, both of which are a type of white blood cell called lymphocytes. B cells produce Abs, which are proteins that bind to specific antigens on the surface of pathogens, neutralizing them or marking them for destruction by other immune cells. T cells, mentioned previously because of their tight connection with the innate immune system, have various roles – some directly kill infected cells ($CD8^+$ T cells), others help orchestrating the adaptive immune response by assisting B cells in their Ab production and regulating immune responses to prevent excessive reactions ($CD4^+$ T cells) (15).

One could view the innate and adaptive immune systems as partners working together to guard our health. The innate system is the rapid, frontline defense, acting almost immediately upon pathogen exposure. In contrast, the adaptive system is slower to respond initially but provides a powerful, specific response and long-term immunity. Since both branches are in many ways interdependent, they must continually work in harmony with each other. The immune system is, in essence, an extraordinarily complex military operation happening within our bodies. With an army of various cells performing different yet coordinated functions. It continuously guards us against a world full of microscopic threats. However, it is essential to note that, like any system, the immune system can sometimes falter or overreact, leading to conditions like immunodeficiencies, autoimmune diseases, and allergies (1). Understanding these aspects further deepens our appreciation for the finely balanced nature of our immune system.

A Tailored Defense: Exploring the World of Antibodies

The journey to our current understanding of Abs, also known as immunoglobulins, has been a fascinating one. It is marked by many pivotal discoveries, resulting in the bestowal of numerous Nobel prizes for medicine (16–19). The initial finding, proving the existence of Abs, dates back to the late 19th century. The credit for this groundbreaking discovery is shared by two scientists, Emil von Behring and Shibasaburo Kitasato. In 1890, they embarked on a series of experiments whereby they injected guineapigs, goats, and horses with diphtheria and tetanus toxins. When

the animals developed an immune response, they drew blood and began an array of experiments. Eventually they were able to extract and identify substances from the animals' blood that could neutralize the toxins. They decided to name these 'antitoxins' (20,21). This was the first hint of what we now call Abs. The significance of this discovery was soon recognized, and von Behring was awarded the first Nobel Prize in Physiology or Medicine in 1901 for his work (22). The term 'Antikörper' (German for 'antibody'), however, was first inadvertently coined by Paul Ehrlich in his much celebrated 'Experimental Studies on Immunity' which were published in 1891 (23). Although the term "antitoxin" remained prevalent among researchers for some time, the usage of "antibody" gradually gained traction and eventually became the more commonly used term. Over the following decades, researchers expanded on these foundations, laying the groundwork for modern immunology.

Abs are central components of the body's immune system. They are proteins produced by specialized white blood cells known as B lymphocytes (also known as B cells). They do so in response to foreign entities, that may pose a threat to our bodies. This includes bacteria, viruses, or other harmful pathogens. The Ab recognizes a distinct molecule on the pathogen, known as an antigen (Ag). The specific binding site on the Ag is referred to as an epitope which amounts a unique molecular pattern. While both TCRs and Abs target antigens, TCRs are restricted to linearized peptide Ags presented by MHC molecules. Ab Ags, however, are far more diverse and can take the form of proteins, peptides, polysaccharides, fats, lipids, as well as nucleic acids. Moreover, Abs can recognize 3-dimensional molecular structures (24–26). These features allow Abs to target specific pathogens with high precision. The role of Abs is to recognize, bind, and help neutralize these foreign substances, playing a vital role in the broader immune defense (1). Here, it's worth clarifying that the term 'neutralization' carries a specialized meaning when it comes to Abs. An Ab can neutralize a pathogen if it can prevent it from infecting host cells. This can occur if an antibody binds to exactly the right portion of a protein that is, for example, used to fuse with and so infect a host cell (27). While some Abs can neutralize a pathogen directly, others merely bind to it without affecting its ability to infect. These are aptly known as non-neutralizing Abs. These cannot neutralize the pathogen because they don't attach to the right region, however, they still play a role in flagging the pathogen for immune cells and can in this way play a key role in immunity (28,29). In contrast, neutralizing Abs can neutralize the pathogen without needing additional help from immune cells (27). Intriguingly, in certain situations, viruses can exploit non-neutralizing Abs – or even insufficient levels of neutralizing Abs – to facilitate their entry into host cells. This phenomenon is called antibody-dependent enhancement (30).

In 1959, Gerald M. Edelman and Rodney R. Porter, working independently, started investigating the precise structure of Abs. Edelman used reduction methods to break down the Ab molecule into smaller components, discovering two large heavy chains and two smaller light chains. At the same time, Porter used enzymatic digestion and

found that the antigen-binding fragment (Fab) could be separated from the crystallizable fraction, or Fc (16,31,32). This work provided insight into the well-known Y-shaped structure of Abs. The Fab portions form the arms of the Y and are responsible for antigen binding. The Fc portion forms the stem and allows the interaction with immune cells. In 1972, both Edelman and Porter were awarded the Nobel Prize in Physiology or Medicine for their significant contributions to the understanding of Ab structure (16). In the ensuing decades we have gained far deeper knowledge regarding the exact structure of Abs. We now know that each chain comprises both constant and variable regions whereby the constant region of the heavy chain can be subdivided into various constant heavy (CH) domains. The variable region (VR) is where Ag-binding takes place, and due to its highly diverse nature, enables Abs to recognize a vast array of Ags. The constant region, on the other hand, determines the class or isotype of the Ab and interacts with other components of the immune system (33) **(Fig. 1)**. The Ab classes, also known as isotypes, include Immunoglobulin G (IgG), Immunoglobulin A (IgA), Immunoglobulin M (IgM), Immunoglobulin E (IgE), and Immunoglobulin D (IgD), each with a specific role and locations within the body (34). For instance, IgG is the most common Ab in blood circulation and provides long-term immunity (35). IgA is the Ab isotype that is produced most in the body overall. It is found on mucous membranes and secretions, protecting body surfaces that are regularly exposed to foreign organisms and substances (36). Furthermore, both IgG and IgA have subclasses, differentiated based on minor structural and functional differences (37,38).

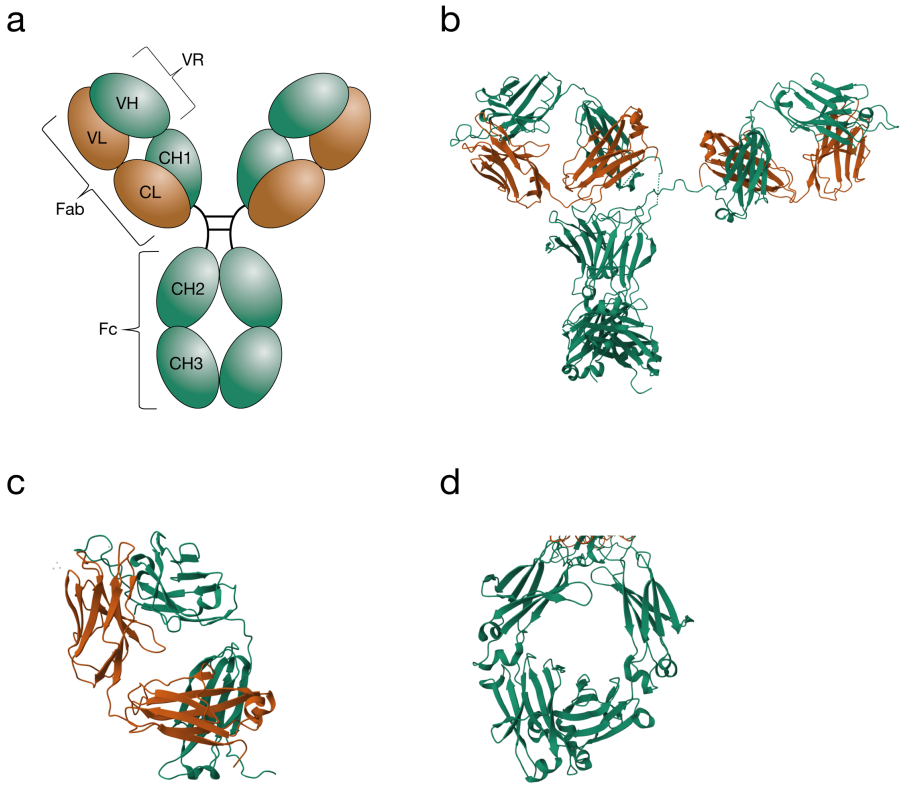


Figure 1
Dissection of an IgG antibody **a** This cartoon depicts an IgG molecule divided into it various regions. The heavy chains are shown in green and the light chains in brown. Labeling indicates the various Ab domains whereby the heavy chain is divided into constant heavy (CH) domains 1-3. CH1 is connected to CH2-3 by the hinge region. The hinge divides the Ab into its Fab and Fc regions.The light chain only has a single constant domain. Both heavy and light chains possess variable regions (VH/VL) which together form the variable region (VR) which determines the binding specificity. **b** 3D Crystal structure of a neutralizing human IgG1 antibody against HIV-1 (39). **c** the Fab and **d** Fc region of the Ab is shown in greater detail. **b-d** The images are from RCSB Protein Data Bank (RCSB.org), PDB ID: 1HZH.

As mentioned above, Abs interact with immune cells through their constant stem or Fc region. This region can bind to specific receptors, called Fc receptors (FcR), found on the surface of various immune cells, such as macrophages, dendritic cells, and neutrophils (40). Each Ab isotype is associated with certain FcRs, whereby IgG primarily interacts with the various Fc gamma receptors (FcγR). The binding of the Fc region of an Ab to an appropriate FcR triggers signaling pathways which allow the immune cell to initiate effector functions associated with the respective FcR. Often Abs are referred to as opsonins whereby other proteins can also function as

such. Opsonins are proteins found outside of cells that attach to specific substances or cells. Once attached, they signal immune cells known as phagocytes to engulf and ingest those substances or cells that have the opsonins bound to them. Therefore, the term opsonization refers to the process of coating a pathogen with Abs, or other opsonic proteins, to tag them for destruction, significantly enhancing their recognition and ingestion by phagocytes. When Abs bind to the body's own cells this can trigger Ab-dependent cell-mediated cytotoxicity (ADCC), wherein immune cells kill the target cells to which Abs have bound. In addition to these processes, the interaction of the Ab Fc region with FcRs can furthermore trigger the release of potent signaling molecules, such as cytokines and chemokines. These molecules can recruit more immune cells to the site of infection, amplifying the immune response (41). It is important to note that the nature of the immune response can be heavily influenced by the specific class of Ab involved, as each class can bind different FcRs and thereby trigger different immune responses (42). For instance, IgG Abs are particularly effective at initiating ADCC, while IgE Abs are known for triggering allergic responses by activating mast cells and basophils (43,44). Thus, the Fc region's interaction with FcRs on immune cells represents a critical link between the pathogen-specific recognition capacity of the adaptive immune system and the potent effector functions of the innate immune system.

For our immune system to effectively identify and neutralize an unimaginably vast array of potential threats, it's crucial that our Abs possess an extraordinary level of diversity in binding specificity. This breadth of specificity enables each Ab to recognize and bind to a unique Ag, allowing the immune system to respond to a wide range of pathogens and foreign substances. This remarkable diversity is primarily generated through two processes: VDJ recombination and somatic hypermutation. VDJ recombination is a genetic recombination of variable (V), diversity (D), and joining (J) gene segments that occurs during the development of B cells in the bone marrow. This process creates a vast repertoire of Abs capable of binding to a multitude of different Ags (45). However, these Abs usually have low affinity (i.e. binding strength) for their targets. After the initial exposure to an Ag, the process of somatic hypermutation further refines this diversity. This process introduces point mutations in the VRs of the Ab genes at a high rate during the proliferation of B cells in germinal centers within secondary lymphoid organs, like lymph nodes. This leads to the production of Abs with higher affinity for the Ag, a process known as affinity maturation (46). Together, VDJ recombination and somatic hypermutation allow our Abs to respond to a remarkably wide range of foreign entities.

After it had been established that Abs can be used as powerful therapeutics in the form of 'antitoxins', researchers were quick to realize that their remarkable specificity could be harnessed to test hypotheses in the lab. Nowadays Abs have become indispensable molecular tools, allowing for the recognition, purification and even modification of targets that are otherwise unperceivable to us. So called

monoclonal antibodies (mAb) represent a powerful tool both in biomedical research and in the clinical treatment of various diseases. Unlike polyclonal Abs, which are a diverse mixture of Abs produced by different B cell lineages, each mAb is produced by identical immune cells that are all clones of a unique parent cell. This means that each mAb is specific to a single epitope allowing for extraordinary precision in targeting (47). The concept of mAbs was first introduced in the 1970s by César Milstein and Georges Köhler, who developed a technique to fuse a normal, Ab-producing B cell with a myeloma cell, a type of cancer cell. This created a hybrid cell, called hybridoma, that could both produce a single type of Ab and replicate indefinitely, providing a renewable source of specific Abs (48). Currently, the generation of mAbs has been refined and several methodologies exist. One widely used approach involves transitory transfection of human embryonic kidney (HEK) 293 cells. In this process, HEK-293 cells, which are easy to grow and manipulate genetically, are transiently transfected with plasmids encoding the heavy and light chains of the desired mAb. This is typically achieved using a method such as lipofection or electroporation, where the plasmid DNA is introduced into the cells. Following transfection, the HEK-293 cells begin to produce the mAb and secrete it into the culture medium. The mAb can then be isolated from the cell culture supernatant (49). This technique offers a rapid and reliable way to produce mAbs with high purity and yield. As we will see in Chapter 2, mAbs have been instrumental tools in some of cell biology's most fundamental discoveries. Today, mAbs have far-reaching applications, from diagnostic tests to cancer therapies.

In conclusion, Abs are pivotal players in the immune response, providing a robust defense against a myriad of pathogens. Their unique structure and diversity enable them to bind specifically to Ags and facilitate their neutralization or destruction by immune cells. The dynamic processes of VDJ recombination and somatic hypermutation ensure an almost limitless adaptability, allowing the immune system to respond to continually evolving threats. As we delve deeper into understanding the complexities of Ab biology, we continue to unlock new ways to harness their potential for therapeutic and diagnostic applications. Indeed, Abs have played a crucial role in all four of the articles included in this thesis.

The Great Cellular Feast: The Role of Phagocytosis in Immune Defense

The journey towards understanding phagocytosis is a fundamental chapter in the field of immunology, as this process represents the first recognized mechanism of the immune response. The concept of "cell eating" was first definitively put forth by the Swiss scientist Albert von Kölliker in 1849. In a publication in the scientific journal, *'Zeitschrift für Wissenschaftliche Zoologie'*, Kölliker meticulously

described the feeding behavior of the heliozoan, *Actinophyrys sol*, a type of amoeba-like alga. He observed the alga engulfing and consuming a small organism, now referred to as a microbe but then referred to as infusoria (50). This was the first recorded instance of such behavior – a process, which we now call endocytosis. The first identification of phagocytosis by leukocytes was made by Ernst Haeckel, a German zoologist, more than 10 years later. Haeckel discovered that the blood cells of a sea slug, known as *Tethys*, were capable of consuming particles of Indian ink or indigo. Although the link between certain blood cells and inflammation in the form of trafficking had already been established many years prior, this was inaugural evidence of immune cells employing phagocytosis (51,52). Haeckel published his findings in 1862 which led to much excitement in the field. In the following years many novel findings began to shed light on the specific morphology of leukocyte movements and ingestions. However, at the time there was still no overarching theory to integrate all this knowledge. It was not until the early 1880s that Élie Metchnikoff, a Russian zoologist, was able to see the larger picture. He named this cellular process ‘phagocytosis’ and the cells that perform it ‘phagocytes’ derived from the two Ancient Greek words 'phagein' (to eat) and 'kytos' (cell) (53,54). While Metchnikoff did not, as many have previously stated, ‘discover’ the process of phagocytosis, he was the first to assign it the role of defending the host against dangerous entities such as microbes. This, along with his discoveries regarding leukocyte recruitment, led to his joint receipt of the 1908 Nobel Prize in Physiology and Medicine, shared with Paul Ehrlich for his work on humoral immunity. Today, he is universally recognized as the father of innate immunity, with some suggesting his influence extends to all immunological sciences (55,56).

Our knowledge regarding phagocytosis has evolved considerably since these initial observations. According to our current understanding, phagocytosis is a fundamental process in the immune response wherein cells engulf large particles ($\geq$ 0.5 μm) such as bacteria, dead cells, or other cellular debris (57). This cellular act of 'eating' is pivotal in maintaining tissue homeostasis and defending the body against foreign pathogens (58). Various types of leukocytes, perform phagocytosis, aptly known as phagocytes. These include neutrophils, monocytes, macrophages, dendritic cells, and even some types of lymphocytes (59). Each has its distinct role within the immune response: Neutrophils act as the first line of defense during infections since they are the most abundant leukocytes and can be rapidly recruited to the site of inflammation from the vasculature (60). Monocytes, circulating in the bloodstream, transform into macrophages when they migrate into tissues, where they continue to perform phagocytosis and play a significant role in the immune system's chronic stages (61). Dendritic cells, primarily stationed in peripheral tissues, are crucial in Ag presentation, making them key players in adaptive immune responses (62). Since phagocytes are akin to microscopic predators, we will henceforth refer to their phagocytic targets as ‘prey’ if the specific target is not relevant.

Phagocytosis can be broadly classified into two forms: opsonic and non-opsonic. In opsonic phagocytosis, the preys are first coated by molecules termed 'opsonins', which include Abs and complement proteins. These opsonins make the process of phagocytosis more rapid and effective (63). For example, the complement system can allow for virtually instantaneous opsonization. In the classical complement pathway, the deposition of complement on a pathogen is triggered by a bound Ab (specifically the isotypes IgG and IgM) (64). The lectin pathway is homologous to the classical pathway except that it is triggered by mannose-binding lectin (MBL) or ficolins instead of Abs (65). These identify specific carbohydrates like mannose or glucose on bacterial surfaces, initiating a cascade that recruits additional complement proteins to serve as opsonins for phagocytes. Finally, there is the alternative complement pathway which is mechanistically distinct from both the classical and lectin pathways. It operates continuously in a low-level activation state, acting as a surveillance system for the body. This pathway does not rely on Abs for initiation; instead, it is spontaneously activated by the hydrolysis of C3 into C3a and C3b. This occurs continuously at a low rate in the plasma and results in free and abundant C3b (66). When this free C3b binds to a pathogen, it sets off the remaining complement cascade. However, C3b is not pathogen-specific and binds to numerous surfaces indiscriminately. To avoid self-targeting, host cells express various regulatory proteins that inhibit the complement activation process (67). Certain complement proteins can act as opsonins through their interaction with complement receptors (CR). These are a group of proteins expressed on various immune cells, including monocytes and macrophages. They play significant roles in the immune response, recognizing and binding to complement-coated pathogens or cells (68). Several complement receptors exist, including CR1, CR2, CR3, and CR4, each offering distinct functions and ligand-binding abilities. For example, CR3 and CR4, discussed further in Chapter 2 and 4, trigger phagocytosis by binding to iC3b - an inactive form of C3b that coats pathogens. Overall, through its various pathways, the complement system can allow for swift and efficient pathogen recognition and targeting. This mechanism plays a pivotal role in immunity, in some cases eliminating the need for a targeted response from the adaptive immune system (69). In fact, in some cases the complement system is even able to kill pathogens directly through large pore-forming complexes – known as the membrane attack complex (70). However, as we will discuss in Chapter 3, group A streptococcus (GAS) has developed a range of complement evasion strategies and is resistant to complement-mediated lysis because of its thick cell wall.

As previously noted, Abs constitute another form of opsonins. They are critical players in the process of opsonization and phagocytosis. Ab-mediated phagocytosis begins when the Fc region of an Ab binds to FcRs located on the surface of immune cells, such as macrophages, neutrophils, and dendritic cells (71). A noteworthy aspect of this mechanism is its ability to target pathogens and entities unknown to the innate immune system. The remarkable diversity and specificity of Abs generated by the adaptive immune response allows the identification and binding of

a vast array of antigens. This means even newly encountered pathogens can be opsonized and flagged for destruction by immune cells, facilitating targeted phagocytosis. Moreover, the therapeutic potential of this process is enormous. mAbs, engineered to target specific antigens, have been utilized to opsonize target cells, such as cancer cells, leading to their phagocytic destruction. This strategy forms the basis of various contemporary cancer immunotherapies. (72,73). Moreover, there is a growing interest in the application of opsonic Abs for the treatment of infectious diseases. Especially since there is growing evidence showing that non-neutralizing, opsonic Abs can also provide protection against pathogens (28,29).

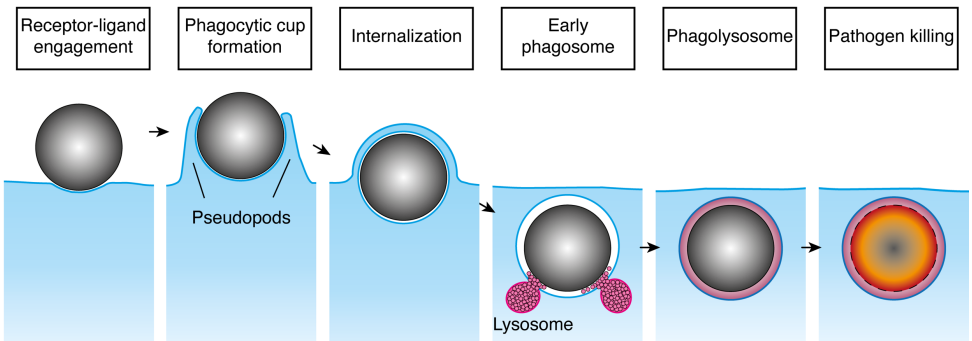


Figure 2
The process of phagocytosis can be divided into various steps. Upon receptor binding, a sequence of cellular rearrangement ensues. Pronounced membrane curvatures appear at the phagocytic cup's base as well as at the starting and ending points of extending pseudopods. This process is driven by significant actin cytoskeletal rearrangements and requires membrane adaptation. Following its formation, the phagosome matures through selective merging interactions with endosomes (not shown) and lysosomes, culminating in the creation of the enzyme-rich, acidic phagolysosome. This environment facilitates the enzymatic destruction of particles or pathogens. This figure best describes the process of macrophage-mediated phagocytosis which is the most thoroughly studied form.

Non-opsonic phagocytosis, on the other hand, doesn't require the prey to be opsonized and usually involves the direct recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) on the phagocyte. These PRRs include: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and Scavenger receptors, among others (74,75). Each of these PRRs recognizes specific MAMPs, which are unique molecular structures that are universally shared among a group of related microorganisms but not present in host cells. This direct interaction between the phagocyte and the pathogen allows the innate immune system to respond rapidly and efficiently to a wide range of microbial threats (71).

Once a prey, has been recognized by a phagocyte, either through opsonic or non-opsonic receptors, the process of phagocytosis is initiated. The actual process of

engulfment can happen through a range of distinct mechanisms that are dictated by the type of receptor and cell type (60). For example, CR-mediated phagocytosis is characterized by so called ‘sinking phagocytosis’ where complement-opsonized preys seem to ‘sink’ into the plasma membrane (76). Given that three out of the four papers included in this thesis examine the impact of specific IgG Abs on phagocytosis, our attention in this chapter will henceforth primarily concentrate on Fc gamma receptor (FcγR)-mediated phagocytosis. This process commences when an IgG-opsonized prey binds to the FcγRs on a phagocyte. This interaction prompts a signaling cascade inside the cell, leading to the activation of proteins that rapidly rearrange the cytoskeleton. This rearrangement, predominantly driven by actin polymerization, propels the membrane to extend around the prey, forming a structure known as a phagocytic cup (77). Eventually, the cup envelops the prey, completely enclosing it within the cell in a vesicle known as a phagosome (**Fig. 2**). This FcγR-mediated mechanism is often described as 'zipper-like' phagocytosis, where the prey is progressively wrapped in the plasma membrane, akin to a zipper closing up (78). Interestingly, it has recently shown that CR3 is involved in FcγR-mediated phagocytosis whereby it was seen to be critical for the regular formation and closing of the phagocytic cup (76) (see **Figure 3** for a detailed description of the hypothetical mechanism). After phagosome formation it goes through the process of phagosome maturation where the phagosome becomes increasingly acidic (from pH 6.5 to 4), gains certain protein markers, and is filled with a variety of degradative enzymes. This occurs through a series of fusions with vesicles in the cytoplasm. The process begins with the fusion of early endosomes, followed by late endosomes, and finally, with lysosomes. The fusion with lysosomes results in the formation of a phagolysosome, where the ingested material is broken down into basic molecular components (79)(**Fig. 2**). It is important to note that this description best exemplifies macrophage-mediated phagocytosis. In particular the process of phagosome maturation can differ between types of phagocytes. For example, the pH of neutrophil phagosomes remains relatively stable throughout (60). Regardless of cell type, mature phagolysosomes can effectively neutralize most potential threats such as bacteria or viruses although some pathogens have developed mechanisms to evade phagolysosomal killing (80–82). FcγR-mediated phagocytosis is furthermore important because it supports the engagement of other immune cells by triggering the discharge of numerous pro-inflammatory cytokines (83).

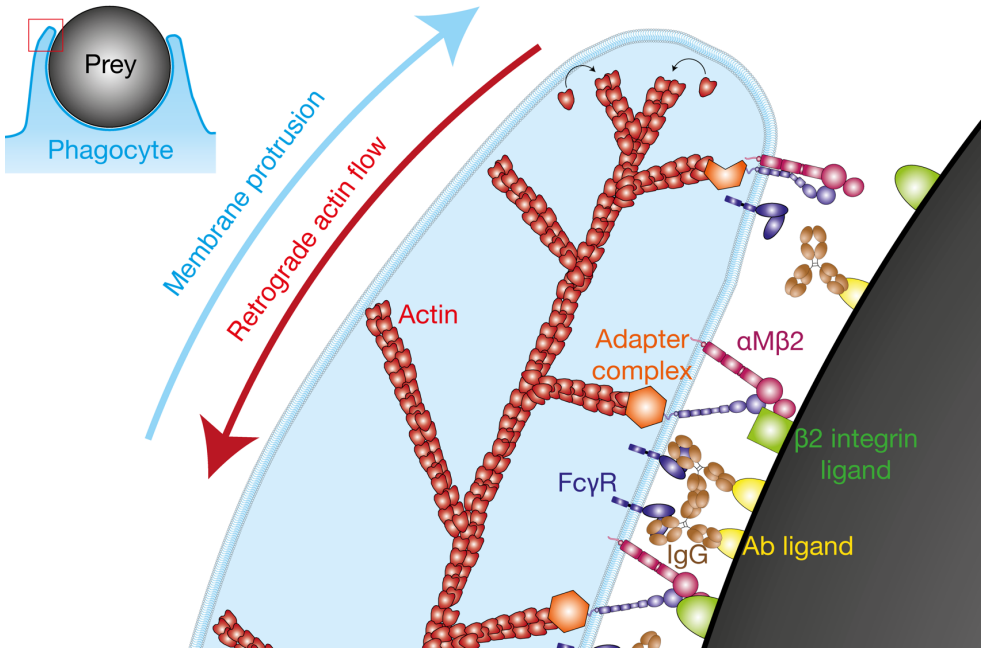


Figure 3
Hypothetical model of αMβ2-assisted FcγR-mediated phagocytosis. Phagocytosis is initiated when an IgG molecule (brown) bound to its target ligand (yellow) on prey (dark grey) is recognized by surface expressed FcγRs (dark blue). FcγR-mediated phagocytosis is characterized by phagocytic cup formation which is driven by polymerization of actin (red). This process, involving the addition of new monomers to the filaments adjacent to the membrane, creates a force that presses against the plasma membrane and results in membrane protrusion. This pressure is equalized between the membrane's protrusion and the actin filaments' retrograde flow. During the formation of the phagocytic cup more prey-bound IgG is recognized by FcγRs on the surface of pseudopod tips. At the same time adjacent αMβ2 integrins (in purple and pink) recognize a diverse array of ligands (green) on the prey. This interaction allows coupling of the actin cytoskeleton with the target prey resulting in mechanical tension. This tension transforms the integrins into an extended-open conformation, which in turn aids in gripping the particle with higher affinity. This tension between actin and integin results in the full formation of the integrin adapter complex (orange), further fortifying the connection and reducing slippage. This enhanced grip on the prey decreases the backward flow of actin, amplifying forward protrusion of pseudopods.This constitutes a possible explanation for why αMβ2 is required for efficient FcγR-mediated phagocytosis. Figure adapted from Jaumouillé et al. 2020 (84).

In conclusion, phagocytosis is a crucial mechanism by which the immune system defends the body against foreign invaders and maintains tissue homeostasis. The process involves several players and steps, from recognition to engulfment and destruction of the target. Whether it's a neutrophil quickly responding to a bacterial invasion, a macrophage cleaning up cellular debris, or a dendritic cell alerting the adaptive immune system, phagocytes are the unsung heroes of the immune system. Furthermore, our understanding of processes like Fc- or CR-mediated phagocytosis has implications beyond basic biology. It contributes to our knowledge of how to modulate the immune response, with potential applications in fighting infections,

autoimmune diseases, and even cancer. The upcoming chapter will review both historical and contemporary techniques for qualitative and quantitative evaluation of this extraordinary process.

Dinner is Served: Techniques for Analyzing Phagocytosis

As a fundamental process of cell biology, phagocytosis is of wide-reaching importance to a number or disparate research fields (85). This makes the precise characterization and quantification of this process ever more important. Over the years, many techniques have been developed, each with their own strengths and limitations. However, an undeniable lack of standardization has been a major obstacle hindering the comparability and reproducibility of results in this field. Here we will shortly review the field of phagocytosis assessment, highlighting the most commonly used methods.

Choosing the appropriate method to study phagocytosis is much like framing a research question in any other scientific field: you first need to carefully define the question you're seeking to answer. This is crucial because phagocytosis is a highly complex and dynamic process, which current technology can only partially capture. Furthermore, the efficiency of phagocytosis can vary depending on several factors, such as sample volume, incubation time, and the ratio of prey to phagocyte – also known as the multiplicity of prey (MOP) (85). Therefore, meticulous planning is essential for any phagocytosis experiment, regardless of the method employed.

Traditionally, Colony Forming Unit (CFU)-based assays have been commonly used and are still used today (86,87). Such methods are performed by incubating phagocytes with live target bacteria. After incubation the sample is diluted and spread on bacterial growth plates (e.g. blood agar). After overnight incubation the number of CFUs per plate is counted. By comparing these with samples where no phagocytes were added it is possible to assess the degree of phagocyte-mediated killing. This method indirectly gauges phagocytosis and is generally used when the primary focus is on the outcome of the phagocytic process, rather than the process itself. Due to these limitations, the method is not well-suited for a detailed examination of the many cellular processes that contribute to phagocytosis. It is primarily applicable for studying bacteria that can be readily cultured using standard bacterial media. Furthermore, these assays can be inconsistent and challenging to execute – especially with bacteria prone to forming chains or clusters, such as GAS.

Microscopy-based methods such as electron- or light microscopy are also commonly used and offer many benefits including the ability to directly visualize the phagocytic process. Often this is done by exposing phagocytes to their prey for

a certain amount of time and freezing the process by adding a fixative. This allows for the retroactive labeling of phagocytes, prey, and even cellular components using dyes and Abs. Intracellular prey can be differentiated from extracellular prey by permeabilizing the plasma membrane of phagocytes or using methods such as quenching or a change in fluorescence triggered by the intracellular environment (88–90). Light microscopy methods offer the benefit that the samples need not be fixed allowing for the capture of timelapse footage of the phagocytic process. In recent years such data has contributed significantly to our understanding of the various phagocytic phenotypes conferred by distinct initiating receptors (76). A major drawback of microscopy-based methods is the ability to scale them. The outcome of experiments is often assessed manually by searching for interacting cells and counting the number of associated or internalized prey. This is highly time-consuming, may introduce observer bias, and cannot be performed in a high-throughput manner. However, this can be accomplished by computer-assisted microscopy since many if not all parts of this process can be automated – from acquisition to image analysis. This kind of methodology has remained elusive for many researchers since it entailed a deep knowledge of programming and engineering. Even experienced programmers have had issues due to the fragmented nature of the various software packages associated with different hardware manufacturers. However, there is hope, global collaboration within the field is beginning to allow the development of software solutions making this technology more accessible for all researchers (91). In addition, recent advancements in automated imaging technologies, drawing insights from the domain of mass spectrometry, are facilitating the capturing of high-precision and high-quality image data. This kind of methodology can moreover provide a comprehensive understanding of the data in the context of a wider population (92).

Flow cytometry is another powerful tool which is commonly used to assess phagocytosis. It allows for the quantification of fluorescently labeled targets and phagocytes in a high-throughput manner. In this method, preys are typically fluorescently labeled, and phagocytosis is then quantified by measuring the fluorescence intensity of the phagocytic cells. Flow cytometry allows the rapid analysis of a large number of cells, providing statistical robustness. It can also differentiate between phagocytic cells and non-phagocytic cells within a mixed population. Some flow cytometers have the option of using multi-well plates which allows for the rapid sequential assessment of samples. This can be especially useful when testing many differing conditions. Nonetheless, like microscopy, distinguishing between prey that have been internalized by the cell and those that are simply adhering to the cell surface can pose a challenge. Techniques akin to those used in fluorescence microscopy may be employed to overcome this issue. For instance, dyes that are sensitive to pH changes can be utilized, as they become more fluorescent when the pH decreases. Yet, their use can be somewhat difficult, as these dyes often have limited stability. This can result in inconsistent increases in fluorescence, making their interpretation difficult. While there are certainly many

challenges to assessing phagocytosis by flow cytometry, the ability to rapidly capture thousands of phagocytic events can offer some significant advantages if the correct experimental setup is chosen (93). This topic will be revisited in Chapter 4 when we discuss Paper I.

For more detailed mechanistic studies of phagocytosis, biochemical assays such as phagosome isolation can be employed. This involves the isolation of phagosomes following particle engulfment and their subsequent analysis to understand the molecular changes occurring during phagosome maturation. This can be achieved using a variety of methods, including the use of prey with certain physical properties such as latex beads. Phagosomes containing latex beads become buoyant in nature and can therefore be easily collected after centrifugation (94). The drawback with this method is that one cannot assess the phagosomes containing prey other latex beads. Therefore, another method has become popular whereby magnetic particles are covalently bound to the prey of interest. After cell disruption this allows for straightforward phagosome purification using magnets (95,96). While these methods can provide detailed molecular insights, they are technically demanding and may not be applicable to all research questions.

To summarize, the choice of method to assess phagocytosis largely depends on the specific research question, available resources, and technical expertise. While each technique has its strengths and weaknesses, a combination of methods is often used to gain a more comprehensive understanding of phagocytosis. For instance, initial high-throughput screening can be complemented by detailed imaging studies or molecular analysis of the phagocytic process. As the field advances, we anticipate the development of novel technologies that will allow even more precise and comprehensive exploration of phagocytosis, enhancing our understanding of this vital immune defense mechanism.

2. Fibronectin

Sticky Business: Fibronectin's Key Roles and Features

Fibronectin (Fn), a high-molecular-weight glycoprotein, is a crucial component of the extracellular matrix (ECM) that plays a pivotal role in various cellular processes (97). The multifaceted nature of Fn becomes clear in a historical context since it was essentially discovered many times over by researchers working in very different fields. This meant that over the years it was designated at least 8 different names (98). This began to change in the early 1970s when a team of researchers, led by Richard Hynes at the Massachusetts Institute of Technology, set out to understand the mechanisms that regulate cell adhesion. At the time, it was known that cells adhere to one another and to the extracellular matrix (ECM), a complex network of proteins and other molecules that provides structural and biochemical support to cells. However, the specific molecules involved in cell adhesion remained a mystery. To uncover these molecules, Hynes and his team employed a novel method. They used mutated fibroblast cells that exhibited defective adhesion characteristics and used them as a model to identify the molecules that normal, adherent cells possess. After analyzing the proteins produced by the fibroblasts, they noticed a large protein that was missing in the transformed, non-adherent cells. This protein, first named ‘large, external, transformation-sensitive (LETS) protein’ was present in the ECM and was found to be critical for cell adhesion (99). Hynes at first thought that he had found a universal molecular switch allowing for cancer development since it was absent in tumor cells, however, this theory was relatively short-lived. We now know that malignant transformation is far more complicated due to the inherently complex nature of cancer. Shortly after Hynes discovery, a number of other research groups independently discovered the protein and even realized that it was in fact the same protein than the cold-insoluble globulin discovered during World War II (100). It was then, in the late 1970s that Vaheri and colleagues from the University of Helsinki finally settled on a common name: Fn (101).

Typically, Fn exists as a dimer, composed of two subunits that are almost identical, each weighing ~250-270 kDa. These subunits are covalently linked by two disulfide bonds close to their C-terminal ends. Every subunit is made up of three different repeating units, known as Fn repeats. These include type I, type II, and type III repeats (**Fig. 4**). Notably, within these repeating units lie crucial binding sites. One

of the most significant is the RGD (Arginine-Glycine-Aspartate) site, a tripeptide sequence found within the 10th type III repeat ($FNIII_{10}$) that is critical for the binding of some integrins. Additionally, there is the synergy site, located adjacent to the RGD sequence (within $FNIII_9$), which amplifies the binding affinity of integrins to the RGD site (102). These critical sites, amongst others, facilitate interactions with other proteins, marking the intricate nature of Fn's role in cellular processes (**Fig. 4**). Here, it is noteworthy that all three types of FN repeats are also present in other molecules, implying that Fn likely evolved through a process known as exon shuffling (103).

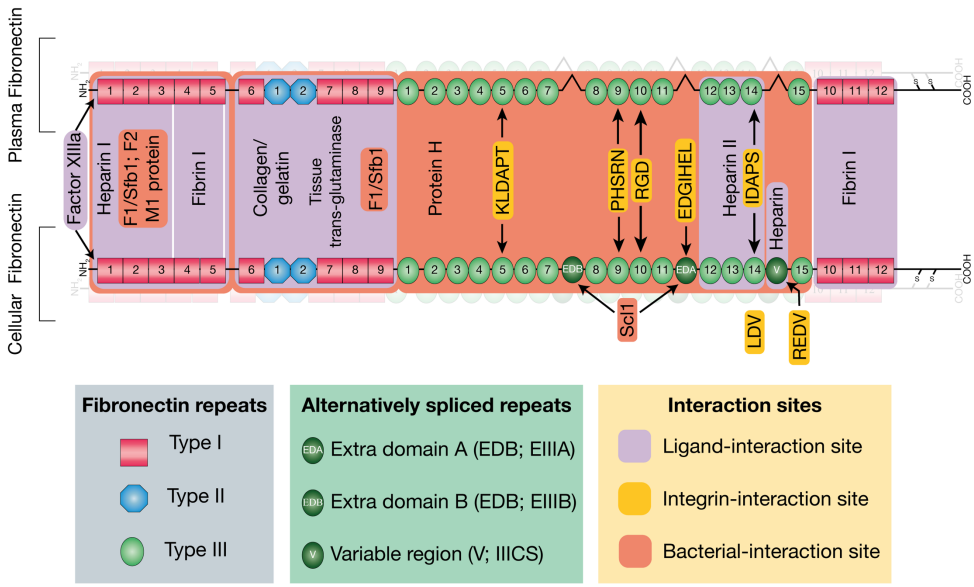


Figure 4
Schematic representation of both a plasma- and cellular Fn homodimer whereby one subunit of each is shown greyed out. Monomers are linked via two C-terminal disulfide bonds. Every Fn monomer is comprised of three distinct repeating units, known as FN repeats: type I (magenta squares), type II (blue octagons), and type III (green ovals). Fn features 12 type I repeats, 2 type II repeats, and 15-17 type III repeats. The top segment of the illustration represents plasma Fn which lacks any alternatively spliced repeats.The bottom segment features cellular Fn. This may encompass two optional FNIII domains (EIIIA/EIIIB) and a single FNIII variable region (V). Interaction sites are highlighted by colored rounded rectangles showing their approximate binding sites within the Fn molecule. Host ligand interaction sites are shown highlighted in lavender and integrin binding sites in yellow whereby these include the specific peptide sequences. Bacterial binding sites are shown highlighted in red.

Even so all Fn molecules are derived from one gene, the ensuing protein can manifest in several forms. This diversity is due to alternative splicing of a single pre-mRNA molecule, potentially leading to as many as 20 different splice variants. A significant splicing event occurs within the central group of type III repeats, from

$FNIII_7$ to $FNIII_{15}$. The inclusion or exclusion of either of the two type III repeats – EDA and EDB – is dictated by whether their corresponding exons are utilized or skipped during the splicing process. Another area of alternative splicing is found in a distinct segment known as the V (variable in length) or IIICS (type III connecting segment) region (104). All these Fn splice variants are roughly categorized as cellular Fn while Fn lacking any of these alternatively spliced regions is referred to as plasma Fn (**Fig. 4**). The exact roles of these various Fn isoforms have, in many instances, remained a mystery. However, in recent years significant progress has been made in unraveling the multifaceted roles of the Fn splice variants. They display distinct distribution patterns in various tissues and are selectively produced in response to different physiological conditions such as growth, wound healing, and differentiation. However, altered splicing of Fn has also been associated with various pathological conditions, including cancer and fibrosis, highlighting the significance of understanding these Fn splicing patterns (105).

Fn's diverse functionality is in part owed to its many binding sites which facilitate its interaction with other ECM proteins, cell surface receptors, and molecules, creating an intricate network that is fundamental to the structural and functional integrity of tissues (102). Importantly, Fn acts as a bridge between cells and their surroundings through its interaction with integrins, a family of cell surface receptors. This connection not only provides physical anchorage but also transduces signals from the ECM to the cells' interior, influencing cell shape, movement, growth, differentiation, and survival. This dynamic interplay is fundamental to cellular behavior and tissue organization (97,106,107). Some of Fn's many interaction partners including ECM ligands, integrins, and GAS virulence factors – their binding sites within the Fn molecule can be seen in **Figure 4**. Fn has been found to be instrumental in a number of physiological processes. For example, during embryonic development, Fn guides cells to their appropriate locations, thereby aiding in tissue formation and organogenesis (108,109). Fn plays a crucial role in hemostasis, as it promotes platelet adhesion and aggregation at sites of vascular injury, contributing to the formation of a blood clot (110). Finally, to enable wound healing, Fn assists in the formation of a fibrin-Fn clot, acting as a temporary scaffold for the various migrating cell types involved in the process of wound healing (111). Despite its many beneficial roles, Fn has a darker side. Its altered expression, organization, or functionality have been associated with various pathological conditions. For instance, an excess of Fn has been observed in fibrotic diseases, where excessive ECM accumulation leads to tissue scarring and organ dysfunction (112). Conversely, reduced plasma Fn levels have been associated with systemic bleeding disorders (110). Furthermore, Fn's role in cancer is complex and multifaceted; it can both inhibit and promote tumor progression depending on the context (113,114).

In conclusion, this versatile glycoprotein serves as a fundamental player in the realm of cell biology. Its complex roles range from supporting embryonic development

and wound healing to maintaining hemostasis and modulating cellular functions. At the same time, dysregulation in Fn pathways can lead to pathological conditions, presenting potential therapeutic targets. As we continue to learn more about this remarkable protein, we gain insight not only into the intricacies of the biological processes it enables but also into novel therapeutic strategies which may in the future be used to combat various diseases.

Fibronectin and Integrins: A Gripping Interaction in Cell Biology

A substantial portion of Fn's function is executed through its interaction with integrins. This group of transmembrane proteins serves as bridges between the extracellular matrix and the intracellular cytoskeleton (115). The discovery of integrins, a family of transmembrane proteins, marked a pivotal moment in cell biology, opening new vistas of understanding into how cells interact with their surrounding environment. The journey of uncovering integrins began in the 1970s with 3 various researchers, Richard Hynes (yes, that Richard Hynes), Erkki Ruoslahti, and Timothy Springer independently making observations that would eventually lead to the recognition of this crucial family of proteins (116).

In the early 1970s the hypothesis had begun to arise that connections must exist, linking the ECM with the actin-based cytoskeleton. By 1976, Fn had been identified as the likely ECM component allowing for this interaction. It was Hynes, who suggested the existence of an ‘integral protein or proteins that might link the two’ (117). This connection between actin and Fn was then independently verified multiple times in the ensuing years (118–120). For several years, the hypothesized "Fn receptor" was viewed as the ultimate pursuit or the 'Holy Grail' within the field (121). It wasn’t until the mid 1980s that some key discoveries allowed for its eventual identification. One crucial step was the development of two mAbs, raised against chicken myoblasts – JG22 and CSAT. Both were found to interfere with the adhesion of avian myoblasts to ECM protein-coated surfaces (122,123). These mAbs turned out to be invaluable tools and were used by Hynes, and many others to isolate the Fn receptors from cell lysates. Another important finding was the identification of the tetrapeptide sequence RGDS as the minimal cell-binding sequence by Ruoslahti. This peptide allowed for the efficient capture and assessment of receptors for both Fn and vitronectin (Vn) (124,125). In the meantime, Hynes had been investigating another lead. He had previously seen that Fn leads to the spreading of platelets. Other groups had further investigated this phenomenon and identified two platelet surface proteins involved in the binding to fibrinogen: Glycoprotein IIb/IIIa (GPIIb/IIIa) (126,127). They soon also found evidence that the same proteins were involved in the binding to Fn suggesting a

common binding site (128). By late 1985 Hynes had successfully cloned and sequenced one of the avian proteins he had isolated with the CSAT mAb. The sequence unveiled a transmembrane protein, showcasing numerous unique and intriguing structural characteristics. It was then that he finally decided to give it a name: integrin. He reportedly chose the name since it described an 'integral membrane protein complex linking the extracellular matrix to the cytoskeleton' (121,129).

In the meantime, Timothy Springer had been studying receptors on the surface of lymphoid and myeloid cells. Although he did not yet realize it at the time, he was also on the verge of discovering integrins. In 1979 he discovered MAC-1 and just two years later he also discovered LFA-1 (130,131). These were both observed to help the cells adhere to and move through the walls of blood vessels – a crucial immune process known as extravasation. These proteins, also heterodimers shared many similarities with GPIIb/IIIa and were even suggested to include the same β subunit (132). Although this was later disproven, it was this paper that brought Hynes and Springer together. At this point Springer had also successfully determined the sequence of the β subunit shared between LFA-1 and MAC-1. It didn't take long for them to realize that this protein was clearly a homologue of Hynes' avian integrin subunit since they shared key characteristic patterns. A few months later, when the sequencing of GPIIb, GPIIIa, and other Fn/Vn receptors was completed it finally became clear that integrins encompass a complex family of transmembrane proteins shared by distant vertebrate classes (Mammals and Aves) (133–136).

The realization that these findings were interconnected was transformative. It turned out that integrins are obligate heterodimers, made up of an alpha (α) and a beta (β) subunit. Remarkably, there are 18 α and 8 β subunits known in humans. These can combine to form 24 distinct integrins, each with unique binding properties and functions (137). Further, it has become clear that integrins are found in all multicellular animals and even plants possess integrin-like receptors – a striking example of evolutionary conservation (138).

The discovery of integrins was fundamental for our understanding of cell biology. These proteins do not merely act as mechanical links between the ECM and the cell's cytoskeleton. They are dynamic structures, transmitting bidirectional signals across the cell membrane (139). On the one hand, they transduce information from the ECM and environment to the cell, guiding processes such as cell migration, proliferation, and differentiation – a process known as outside-in signaling (140). On the other hand, they communicate the cell's status to the outside, altering their adhesion and migration properties in response to changes in the cell – so-called inside-out signaling (141). Furthermore, integrins are crucial for the assembly and remodeling of the ECM, impacting tissue development, maintenance, and repair. They are also involved in various pathological processes, including inflammation, autoimmune diseases, and cancer metastasis (142). Hence, understanding these

interactions at a molecular level could reveal potential targets for therapeutic intervention.

Among the array of integrins, the beta-2 (β2) subfamily stands out since they are exclusively expressed on leukocytes (143). These integrins primarily interact with cell adhesion molecules on the surface of other cells rather than the extracellular matrix (144). Beta2 integrins are crucial in mediating immune cell adhesion, migration, and activation, which explains why they need to recognize a wide range of ligands (143). The integrin αMβ2, (also known as CR3, MAC-1, or CD11b/CD18) has many times over been shown to interact with Fn (145–147). While there are findings indicating that the αMβ2-Fn interaction is critical for respiratory burst in neutrophils (148) there is still an overall lack of evidence regarding the physiological significance of this interaction. It is, however, plausible that it is particularly important during inflammation, where Fn is often upregulated.

To conclude, the interaction between Fn and integrins represents a cornerstone of cell biology. It allowed scientists to understand the dynamic nature of cell adhesion as well as the intricate crosstalk between cells and their environment. This, in turn, has had profound implications for our understanding of embryonic development, immunology, and disease. While initially identified as leukocyte and Fn receptors, integrins have now emerged as versatile proteins interacting with a multitude of ligands, with Fn being one of the key partners. The 'promiscuous' αMβ2 integrin exhibits a unique adaptability in its ligand recognition, and its interaction with Fn seen under certain circumstances adds another layer of complexity to this dynamic relationship. The interaction between αMβ2 integrin and Fn will be further discussed in Chapter 4 regarding the findings in Paper IV.

Bacterial Fibronectin Binding Proteins: When Host Proteins Go Rogue

In the dynamic interplay between host and pathogen, bacteria have evolved intricate strategies to exploit host systems to their advantage. Fn has emerged as a notable target of several bacterial pathogens. These have devised mechanisms to bind to Fn and utilize it as a bridge for adherence to host tissues, facilitating infection, and evading host defense mechanisms (149,150). Interestingly, specifically a wide range of host-associated bacteria have evolved Fn-binding proteins (FnBP) that allow them to bind Fn and exploit its functions. FnBPs are expressed on the surface of both pathogens and commensals, facilitating the interaction between bacteria and host cells. In pathogens, certain FnBPs enhance virulence by aiding in the attachment to host cells, epithelial cell invasion, and disruption of signaling pathways. While FnBPs in non-pathogenic commensal strains do not suffice to

cause virulence, they play a vital role in enabling these organisms to attach to and remain within their specific bodily niches.

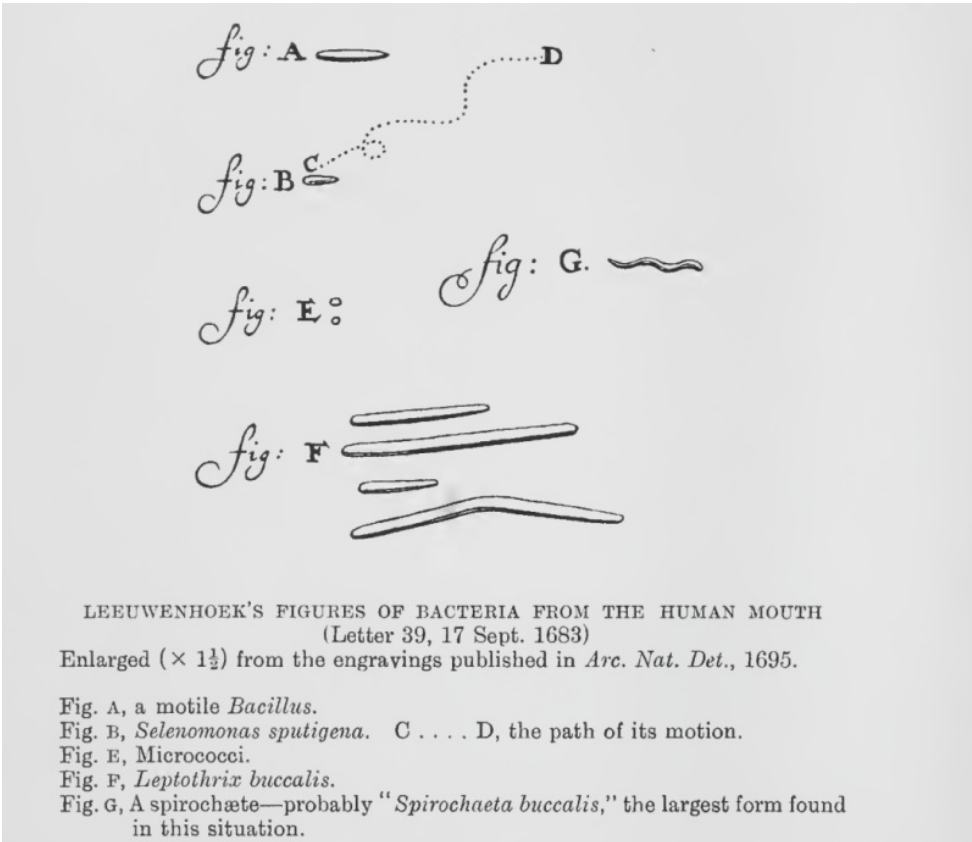
Over 100 FnBPs have been identified across various bacterial species, highlighting that Fn binding is an essential, evolutionarily conserved feature. These FnBPs exhibit great diversity and can bind Fn through various mechanisms. Some bind to the so-called 'canonical' bacterial Fn binding site located in the modules FNI_{1-5}. This region is also required for the binding of Fn to heparin, fibroblasts, and fibrin (**Fig. 4**). It earnt this name since it was the first identified bacterial binding site on Fn and many FnBPs from a number of bacteria bind to it – especially Gram-positive cocci. This binding occurs with the so called canonical Fn binding repeats (149). For example, the homologous FnBPs F1 and Sfb1 expressed by certain GAS strains contain such repeats (151). However, these were later shown to also contain a region recognizing the FNI_6-FNI_9 region (152). After the initial binding interaction between canonical FnBPs was characterized a number of novel FnBPs with distinct binding patterns emerged. These FnBPs lacked the characteristic Fn binding repeats and interacted with other segments of the Fn molecule. For example, the M-like protein, Protein H, was found to bind to the Fn via the FNIII repeats instead of FNI (153). Another example is the 54-kDa protein Fbp54 which lacks all typical Fn-binding sequences. While it was initially identified in streptococci, divergent homologs of this protein have been discovered in a wide range of host-associated bacteria. These include streptococci, lactococci, lactobacilli, clostridia, listeria, pneumococci, enterococci, and bacilli (149). The identification of these unique FnBPs allowed for a fresh perspective where bacterial proteins, previously known for other functions, also serve as FnBPs (149). These multifunctional proteins are especially interesting because, although it has been possible to approximate the binding site on the Fn molecule, the actual amino acid sequences within Fn and the FnBPs have often remained elusive. At this point, most portions of the Fn molecule have thus far been identified as potential binding sites for FnBPs (**Fig 4**) – a testament to the degree of adaptation between bacteria and host. Finally, the M1 protein serves as another striking example of this protein multifunctionality. Not only does it possess the ability to bind to Fn, it also exhibits several other functions such as binding to fibrinogen (Fb), IgG, and glycosaminoglycans (GAG) (154–156).

The interaction between Fn and bacteria offers an illustrative example of how pathogens as well as commensals use host systems to promote their survival and establishment within the host. Understanding these interactions is crucial, as it may provide novel targets for therapeutic intervention against pathogens. The fascinating world of bacterial interactions with Fn is a testament to the complex battle between host, pathogen, and commensal. A dance of survival that continues to shape the evolution of all parties involved. The M1 protein and other GAS virulence factors will be further discussed in Chapter 3.

3. Group A Streptococcus (GAS)

GAS Chronicles: From Childbed Fever to Science's Frontiers

The knowledge of illness caused by streptococci dates back many centuries. References to the illness 'erysipelas' (red skin) as well as the symptoms of childbed fever can be found in Hippocrates' ancient texts from the 4th century BC (157). It wasn't until the 16th century AD that these diseases were then divided into separate health conditions. It was Giovanni Filippo Ingrassias, a Sicilian anatomist and practitioner, who first described a disease termed "rossalia" that was characterized by 'numerous spots, large and small, fiery and red, of universal distribution, so that the whole body appeared to be on fire' (158). Numerous theories about the origin of diseases arose in the subsequent centuries. People associated their emergence with astronomical events such as comets and eclipses or believed in the transmission of diseases via odors and vapors. Significant strides in understanding the causes of diseases weren't made until the 17th century. It was then that Anton van Leeuwenhoek, a Dutch draper, became interested in experimenting with lenses to improve thread quality assessment. These experiments led to the creation of ever more powerful lenses which eventually allowed him to see into the microbial realm. His impressive microscope allowed for the identification of previously unknown life forms. These 'little bloodless animals', as he described them, were later identified as bacteria, and included cocci, bacilli, and spirochetes (**Fig. 5**) (159,160). This marked a significant breakthrough since it was in 1736 that a Dutch surgeon, treating a patient with a wound infection, correctly attributed it to 'Leeuwenhoek's little animals' and found that they were killed by treatment with mercury (161).



LEEUWENHOEK'S FIGURES OF BACTERIA FROM THE HUMAN MOUTH
(Letter 39, 17 Sept. 1683)
Enlarged (× 1½) from the engravings published in *Arc. Nat. Det.*, 1695.

Fig. A, a motile *Bacillus.*
Fig. B, *Selenomonas sputigena.* C . . . . D, the path of its motion.
Fig. E, Micrococci.
Fig. F, *Leptothrix buccalis.*
Fig. G, A spirochæte—probably "*Spirochaeta buccalis*," the largest form found in this situation.

Figure 5
A figure included in a letter sent by Leeuwenhoek to the Royal Society in 1683. The figure shows drawings by Leeuwenhoek of bacteria from his mouth; the dotted line portrays movement (162). Copyright © The Royal Society.

Although bacteria's existence was now established, their role as disease-causing agents wasn't yet accepted by the scientific and medical communities. It was not until 1842 that a young Hungarian physician, Ignaz Semmelweis, began working in the obstetric wards of the Vienna and saw a connection between sanitation and deadly disease. He noticed that women giving birth with the assistance of physicians and medical students were almost 10-times more likely to die than those who were attended to by midwives. He observed that the physicians and medicals students first went to the participate in autopsies and did not wash their hands before attending to their patients. This resulted in his hypothesis that the increased rates of childbed fever, also known as puerperal fever, were caused by the physical transmission of a disease entity from cadavers to the patients. In 1847 he ordered all medical personnel to adhere to strict sanitary guidelines including the washing of hands in a chlorinated lime solution. This resulted in a remarkably reduced mortality

rate due to puerperal fever – from 11.4% to 2.7%. Semmelweis had accomplished an impressive feat; by making astute scientific observations he was able to save many lives and even pioneered the use of statistics to prove his hypothesis. Nevertheless, he was shunned and mocked by his immediate superiors and eventually returned to Budapest where he later, in 1861, published his findings (163). Despite his extraordinary accomplishments, it was not until 14 years after his death in 1865 that his findings were fully recognized by the scientific establishment (164).

It was the Austrian surgeon Theodor Billroth who first formally identified streptococcal microbes in cases of erysipelas and wound infections in 1874. He described 'small organisms (Kettenkokken) as found in either isolated or arranged in pairs, sometimes in chains of four to twenty or more links (Streptococcus; Greek: strepto, a chain, and coccus, a berry).' (165). These findings were extended when Louis Pasteur was able to isolate the microbes from the uteruses and blood of women with puerperal fever. For the first time there was proof that these bacteria were the causative agent behind the disease that resulted in the highest mortality rates among women and newborns (166). The nomenclature was further refined by Friedrich Julius Rosenbach in 1884, when he analyzed bacteria from pus-filled wounds and named the species *Streptococcus pyogenes*, deriving from the Greek words for 'pus forming' (167). The differentiation of streptococci was further enabled by the invention of blood agar plates by Hugo Schottmüller in 1903 (168). This was possible since the various types of streptococci interacted differently with the blood resulting in varying forms of hemolysis. These could be seen as hemolytic rings around the bacterial colonies. Streptococcus pyogenes was identified as being beta-hemolytic, meaning that the blood agar would become clear.

Around the same time, Dochez, Avery, and Lancefield began experimenting with immunological methods to show variances among *S. haemolyticus* strains (169). In 1933, Lancefield subdivided streptococci into groups A through X based on their surface antigenic differences (86). The categorization of groups was based on the animals affected by the strains. As *Streptococcus pyogenes* infects humans, it was classified under Lancefield group A, thereby earning its alternative name: group A streptococcus. The continued use of this terminology has been criticized by some since in reality it does not fully distinguish S. pyogenes from other streptococcal species – the cell walls of both *S. dysgalactiae* and *S. anginosus* can possess the A antigen as well. However, many consider the debate of 'correct' terminology redundant since the designations 'group A streptococcus' and '*Streptococcus pyogenes'* have essentially become synonyms and no other streptococci are generally referred to as such. More recently, the differentiation between various GAS strains has become routinely possible through molecular methods, such as emm-typing. This is done by using polymerase chain reaction (PCR) to amplify the gene that encodes the M protein (emm) which varies between strains (170,171). So far over 240 distinct emm types have been identified (172). This has allowed for a

better understanding of the epidemiological prevalence of specific emm types and their global tracking (173). This extraordinary level of diversity amongst emm genes has inevitably led to pitfalls – especially regarding largescale epidemiological analyses and vaccine development. This has prompted the development of novel classification methods that are not solely based on the genetic code of one gene (emm). One such approach is the clustering of the various emm types based on genetic as well as the functional characteristics of the encoded M protein (174). Moreover, the advancements in sequencing technologies have made techniques like multilocus sequence typing and whole genome sequencing more affordable and accessible (175,176).

Group A Streptococcus: An Exceptionally Successful Microbe

GAS is, in part, such a fascinating organism to study due to the astounding level of host-adaptation it has achieved. This adaptation becomes more fascinating given that GAS is the sole pathogenic streptococcus species known to exclusively infect humans (177). This means that its success as a species is fully dependent on the continuous infection of and transmission between humans. This narrow host adaptation is uncommon and could have spurred species diversification, as most pyogenic streptococcal species have the ability to infect a broad spectrum of mammals (178). It is now believed that the ability to infect humans as well as the emergence of GAS as a distinct species was likely made possible by the acquisition of the M-protein island, which introduced 35 universal GAS genes. These include the secreted pyogenic exotoxin SpeB and its regulator RopB. These genes are thought to have limited the bacteria's virulence and aided in the colonization of the nasopharynx (178,179). Yet, it does seem odd that bacterial evolution would result in the restriction to a single host reservoir. One would imagine that bacteria with a broad range of hosts would have more opportunities to proliferate and thus have an evolutionary advantage. Conversely, GAS is one of the most successful human pathogens, infecting more than half a billion people every year (180). Was it this newfound ability to stably colonize the nasopharynx that made GAS such an exceptionally successful species?

When GAS infects a host, the ensuing host-pathogen interaction can be roughly divided into 3 categories:

Invasive: this includes severe, life-threatening diseases such as **necrotizing fasciitis** (commonly referred to as the flesh-eating disease, is a bacterial infection that leads to the decay and death of the body's soft tissue (181)), **sepsis** (also known as blood poisoning, is a critical health condition that occurs when the body's reaction to an infection inflicts damage on its own tissues and organs (182)), and

streptococcal toxic shock syndrome (STSS is characterized by a sudden onset of shock, multi-organ failure, and a rash that can lead to skin peeling. It is one of the most serious and life-threatening complications of GAS infections (183)). These infections are very rare and make up roughly 0.001% of all symptomatic infections (184).

Symptomatic superficial: this category includes diseases that are less severe and generally self-limiting in nature. Examples include **pharyngitis** (commonly referred to as strep throat; pharyngitis refers to the swelling of the pharynx, the area at the back of the throat, and is commonly characterized by symptoms like a sore throat and fever (185)), **impetigo** (an infection affecting the outer layers of the skin, often manifesting as yellow crusts on the face, arms, or legs (186)), and **scarlet fever** (refers to a clinical condition marked by the occurrence of a diffuse erythematous rash in conjunction with a superficial GAS infection, typically pharyngitis (187)). These infections are common, representing over 99% of all symptomatic infections (184). While these infections generally resolve spontaneously, if left untreated they may lead to immune-mediated disorders including **acute rheumatic fever** and **acute post-streptococcal glomerulonephritis**(188). The former is especially problematic since it can result in the development of **rheumatic heart disease** (RHD) (189,190).

Asymptomatic carriage: GAS can also infect humans without causing any symptoms. This is especially common amongst schoolchildren where pharyngeal colonization rates have been seen to reach as high as 15–26% (191,192). Though GAS-related disease burden is high, the observed high rates of asymptomatic carriage in the population have even led to suggestions that GAS is an opportunistic pathogen rather than an obligate one (193).

These various forms of infection can occur consecutively or independently. The characteristics of an infection are determined by an interplay between the infectious agent and its host. The presence and relative frequency of the various types of infections can thus be attributed to variations in either or both the pathogen and the host (194). Although many studies have primarily placed an emphasis on invasive infections, it is important to remember that they only constitute a very small portion of all infections (180). Moreover, the distribution of global GAS infections is highly unequal with a disproportionate share of the cases occurring in developing nations, within indigenous communities, and in economically disadvantaged areas of developed countries (195). Children and adolescents are at a higher risk for GAS infections, with the peak incidence of strep throat occurring between the ages of 0 and 15 (196). Other groups at risk include individuals with underlying health conditions (197), the elderly (198), and pregnant as well as post-partum women (199). GAS is primarily transmitted through respiratory droplets or direct contact with infected individuals. This means that any instance which results in the dense gathering of people is associated with higher risks of infection. Outbreak-prone areas have been found to include nurseries, schools, elderly care homes, military

bases, and homeless shelters (200–202). This explains why outbreaks follow seasonal patterns in temperate climates. In the colder months people tend to congregate indoors, increasing the likelihood of close contact and transmission. Evidently, the factors that increase GAS transmission disproportionately affect and sicken already vulnerable and disadvantaged populations. Further, it is exactly these communities that have limited healthcare access, meaning that more of these infections remain untreated. In fact, 97% of all documented yearly GAS infections occur in the Global South and these figures are likely an underestimation since many cases likely go undocumented (195,203,204).

For many years the main focus of GAS research was focused on invasive infections. This is likely because superficial disease caused by GAS had essentially become a matter of no concern in the Global North. Continuous access to health care, nutritious food, and adequate housing are just some of the factors that have reduced the disease burden of high socioeconomic status populations. Perhaps most importantly, this has led to a steep decline in RHD cases in these areas. While superficial infections are generally self-limiting, guidelines recommended that they be treated with antibiotics. This is because, amongst other reasons, it has been shown to prevent rheumatic fever which can lead to RHD (205,206). In fact, it seemed as if RHD had almost been forgotten as a direct consequence of GAS infection while simultaneously the disease was leading to a staggering amount of mortality and morbidity in resource-limited settings. Further, until recently it had been assumed that only a small subgroup of emm type strains can cause RHD. This meant that emm types were classified as either rheumatogenic or non-rheumatigenic depending whether they contained 'rheumatogenic motifs'. However, initially only 10 of the over >240 currently identified emm types were thought to be rheumatogenic. This theory is now being challenged with many more emm types being found to be associated with RHD than previously assumed (207). When regarding the global prevalence rates of death and injury caused by RHD, a depressingly clear pattern emerges. Western European countries have particularly low rates. All but a few outliers have rates below 0.05 per 1000 children. In stark contrast, many countries in Sub-Saharan Africa show rates more than 150-times as high (208) (**Fig. 6**). A study from 2019 estimated that RHD and RHD related disorders led to almost 3-times more deaths than invasive GAS infections (209). This doesn't even take into account the over 10 million disability-adjusted life years caused by RHD (210). In the past years, progress has been made in the academic sphere, where many publications have highlighted the neglected burden of RHD and how it is intensified by global wealth inequities (211–216). Suggested approaches designed to prevent or treat GAS infections should be practical, readily available, and cost-effective, particularly in areas with limited resources (217). At present, there is no approved vaccine available to manage GAS infections, but efforts are being made to create one (218). The advancement of such a vaccine has encountered obstacles, including the existence of numerous distinct GAS serotypes, changes in antigens within a single serotype, safety issues, and a lack of agreement on clinical endpoints needed

to establish proof of concept (219). Research suggests that a vaccine could be instrumental in decreasing the prevalence of these diseases, strongly advocating for its development and implementation (219–221). Finally, the World Health Assembly recently unanimously adopted a Global Resolution calling for better control and prevention of GAS infections and RHD (222). This signals a newfound understanding for the gravity of the situation – especially amongst high-income countries. Overall, significant advancements are being realized across basic scientific, clinical, translational, and population-level research. The answers to these challenges are within reach, and with sufficient funding, we have the potential to considerably diminish the worldwide impact of diseases caused by GAS within our generation.

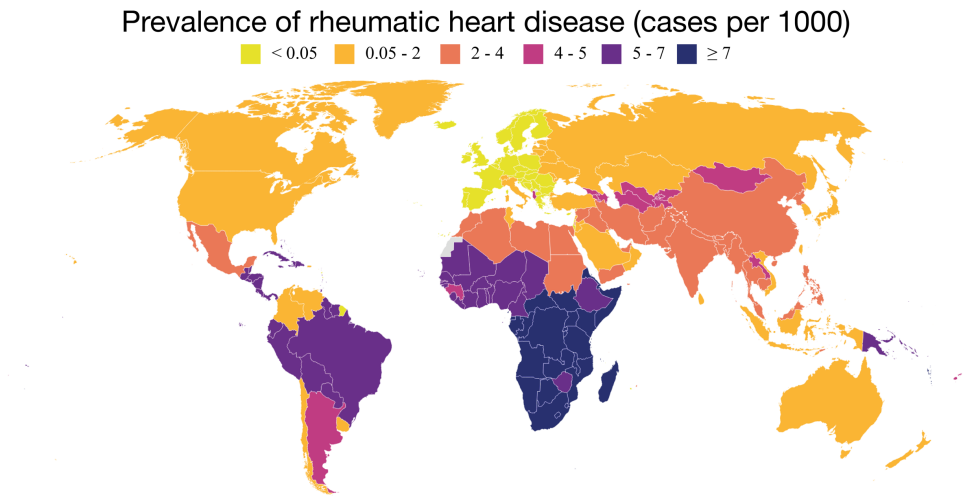


Figure 6
Prevalence of rheumatic heart disease resulting in injury or death in children aged 5-14 in the year 2019 (cases per 1000). No data was available for countries shaded in grey. The data is derived from the Global Burden of Disease Collaborative Network (208) and the map was created with Datawrapper.

GAS Virulence Factors: A Formidable Arsenal

The nasopharynx presents a particularly difficult environment for bacteria to thrive in. Challenges include a mucus layer that hinders the bacteria's ability to attach to epithelial cells, a scarcity of nutrients like glucose, other commensal bacteria which compete for these nutrients, and considerable environmental fluctuations concerning temperature, oxygen, and pH levels. All things considered, it makes sense that GAS would need to be highly adapted to accomplish stable infections within such a niche and even thrive whilst doing so. Much of this adaptation comes

in the form of an extensive array of virulence factors (VF) that GAS deploys. These specialized proteins bolster the bacterium's abilities to not only initiate an infection but also establish a stable foothold within this harsh, unforgiving niche. To provide an overview I have decided to divide these VFs into 4 categories, whereby these are not always mutually exclusive:

Adhesins: These factors include various surface proteins that allow GAS to attach to host tissues, recognizing and binding specifically to host components such as Fn, collagen, and laminin (223). Examples of these include pili, M protein, Fn-binding proteins, and lipoteichoic acid (LTA). The importance of these adhesion virulence factors lies in their essential role in colonization and infection by anchoring the bacteria to host cells and the ECM. It is theorized that the initial attachment of bacteria occurs in a two-step process. It begins with weak and/or long-range interactions and is then followed by more specific and high-affinity binding (224). According to this hypothesis cell wall-bound LTA combined with far-reaching pili are able to facilitate initial, weak adhesion to host components. Once GAS has been brought into closer proximity to host cells and the ECM the second stage of adhesion can begin. This stage involves the occurrence of multiple high-affinity binding events such as protein-protein or glycan-protein interactions. One such high-affinity binding mechanism could include the M1 protein's ability to recognize and bind to the ABO(H) blood group antigens, which are found abundantly expressed within the nasopharyeal niche (225). Moreover, many adhesins are also known as ‘invasins’ since they have been found to allow GAS to become internalized into epithelial cells where they can remain hidden from the immune system (226). Another function of these proteins extends beyond tissue adhesion to the binding of functional host proteins on the bacterial surface, a process that can be exploited to benefit the bacterium.

Enzymatic: These secreted, enzymatically active VFs are numerous and crucial for both initiating infection in the host and maintaining it over the long term. For instance, the streptococcal cysteine proteinase (SpeB) stands as one of the most extensively researched GAS virulence factors. As a broad-spectrum protease, it has the remarkable capability to cleave over 200 host proteins, affecting both immune defense-related proteins and extracellular matrix (ECM) proteins. This dual function means it is vital for both the persistence and spread of the bacteria within the host (227). Other examples include streptolysin O (SLO) which is an enzyme that can lyse a broad range of host cells, as well as NAD^+ glycohydrolase (NADase) which acts synergistically with SLO to cause severe cytotoxicity (227). In fact, it has recently been shown that NADase activity can greatly influence disease severity by interfering in the innate immune system’s capacity to sense bacterial second messenger molecules (228). Since these enzymatic VFs are differentially expressed, they can be essential in determining the virulence of certain GAS isolates (229).

Toxins: GAS toxins are essential to the virulence and pathogenesis of infections caused by GAS. Notably, there is significant overlap with the previous category, as

many of these toxins possess enzymatic activity – the most prominent example being the aforementioned mentioned SpeB. However, there are a number of other Spe variants which are not enzymatically active whereby SpeA and SpeC are best studied. These secreted exotoxins are known as superantigens (SAgs), whereby SAgs are characterized as potent immunomodulatory proteins which can trigger a non-specific, excessive immune response and have been shown to be linked to severe disease outcomes (230,231). After secretion by GAS, SAgs are initially taken up by APCs which present them on their surface via MHC class II molecules. The particular structure of these toxins means that they can be recognized by a wide range of TCRs by engaging their lateral surfaces (232). This leads to an unspecific activation of a large portion of the body's T cells, triggering the release of inflammatory cytokines. This fulminant release of cytokines is partly to blame for conditions such as scarlet fever, sepsis, and STSS (233). Moreover, this promiscuous activation of T cells by SAgs can make these hyporesponsive and less capable of mounting an appropriate immune response to the pathogen (234). These SAgs are differentially expressed between isolates and have been linked to certain prophages carrying the necessary genes (235). GAS also secretes the widely studied toxin streptolysin S (SLS). This is a non-enzymatically active cytolytic toxin that can lyse a wide range of mammalian cells and is responsible for the typical β-haemolysis pattern which appears around colonies grown on blood agar (236). Finally, the multifunctional M protein can also function as a toxin. This occurs when it is cleaved from the bacterial surface by bacterial of host proteases (237). Especially soluble M1 has been widely studied and has been shown to activate neutrophils, monocytes, and T cells. This activation is, in fact, so potent that some have suggested it be considered a novel SAg (238). Overall, the importance of these toxins lies in their ability to aid the bacteria in evading the host's defense mechanisms and contributing to the severity and progression of disease.

Anti-phagocytic factors: It is important to recognize that these factors largely fall into the initial three categories. However, due to the abundance of GAS virulence factors (VFs) that specifically resist phagocytosis, I believe they warrant special mention. These include a collection of anti-phagocytic proteins that contribute to the ability of GAS to evade phagocytosis by host phagocytes. This protects the bacterium from phagocytic killing which represents the main host clearance mechanism for extracellular pathogens. This enhances the bacterium's ability to infect and persist within a host (226). A specific mechanism involves an M-like protein, known as protein H, which can bind the human complement inhibitor C4BP. This binding results in reduced complement deposition on the bacterial surface, thus decreasing the ligands available for phagocytic CRs (239). M protein, which will be discussed in more detail in the next chapter, has also been shown to exhibit anti-phagocytic properties through similar mechanisms. Notably, both these examples fit into both the adhesin and anti-phagocytic categories, as they include the sequestration of a human protein. Another example is the cysteine protease IdeS which specifically targets and cleaves human IgG. Since this cleavage occurs just

below the IgG hinge region this results in F(ab')$_2$ fragments lacking Fc domains. These Abs will still bind but can no longer serve as opsonins for phagocytes (240). Similarly, the endoglycosidase EndoS is a protein that can specifically cleave the N-linked glycan from IgG. This loss of glycosylation has a substantial effect on IgG-mediated effector functions including phagocytosis and complement deposition (241,242).

It is clear that GAS' pathogenicity can largely be attributed to its diverse array of virulence factors. In this chapter, we've highlighted only a selection of them, as covering the full spectrum would be beyond the scope of a single chapter. It's worth noting that GAS also boasts virulence factors that don't conform to the four previously described categories. A prime example is the secreted virulence factor, streptokinase, which has been explicitly linked to virulence. Within the host environment, streptokinase serves to transform the host's inactive zymogen plasminogen into its active form, plasmin. Interestingly, this transformation doesn't occur through enzymatic action but instead is achieved by inducing a conformational shift within the target protein. As an enzyme, plasmin has the ability to break down blood clots and the ECM, effectively clearing the way for bacterial dissemination. This unique mechanism has been demonstrated as vital in facilitating the spread of GAS to various other locations within the host (243). Interestingly, certain GAS M proteins have evolved the capability to bind plasminogen. This mechanism secures the zymogen in proximity, ensuring a robust surface presence of plasmin that can be exploited by GAS. This particular function has been identified as vital for virulence in animal studies (244). This demonstrates yet another instance where GAS's VFs operate synergistically, reflecting a finely tuned equilibrium that has arisen through millennia of co-evolution with the human host. In the following chapter, we will examine the GAS M protein in greater detail – a fascinating molecule that has, in many respects, become emblematic of this pathogen.

The Streptococcal M Protein: An Intriguing Enigma

The M protein is one of the key virulence factors produced by GAS and plays a crucial role in the pathogenicity of this bacterium. It is found projecting approximately 500-1000 Å from the GAS surface in the form of hair-like structures (245,246). This means that the M protein is ideally positioned to engage with host components and play a multifaceted role in GAS infections. M protein aids in GAS' adhesion to host cells (225), intracellular invasion (247), formation of bacterial aggregates that boost resistance to phagocytosis and enhance host cell binding (248), counteracting antimicrobial peptides (249), triggering clotting and severe inflammation (250), and establishing GAS biofilms (251). M protein is also well known for preventing Abs and complement from being deposited on the bacterial surface, allowing GAS to circumvent leukocyte-mediated phagocytosis and thrive

in the host (252). Importantly, it is also implicated in triggering Abs that can cross-react with host proteins leading to autoimmune responses such as RHD (253). This has presented a significant obstacle to the creation of a GAS vaccine based on the M protein (254). The numerous roles of M protein can be explained by its interactions with numerous host elements. For such a versatile protein, M protein showcases a seemingly straightforward α-helical coiled-coil structure. Intriguingly, protein structural studies have shown that, not only does the M protein constitute a nonideal, instable α-helical coiled-coil, but they have also shown that its nonideal nature is crucial for its functionality (255).

The M protein is generated in its immature form, possessing an N-terminal signal sequence and a C-terminal processing site. This N-terminal signal sequence is highly conserved between M variants and guides the M protein to the bacterial division septum where it is then cleaved by a signal peptidase (256). Next, M protein is secreted through the bacterial membrane where it is processed by the enzyme sortase. This first cleaves the protein at the "LPXTG" motif between the Thr and Gly. Then it covalently anchors the Thr residue to the peptidoglycan precursor lipid II, and eventually to the peptidoglycan itself (257). This results in a short cell wall spanning domain. After both ends are trimmed, the mature M protein is found as a homodimer made up of two identical strands. These both typically consist of around 300 to 400 residues (**Fig. 7**). As mentioned previously, the M protein can also be found in a soluble form. This detachment can result from the action of the streptococcal enzyme, SpeB, or from neutrophil proteases during infection (257). Notably, despite being cleaved, the protein maintains its inflammatory and coagulation-inducing attributes and can be one of the driving forces behind severe conditions such as STSS (258).

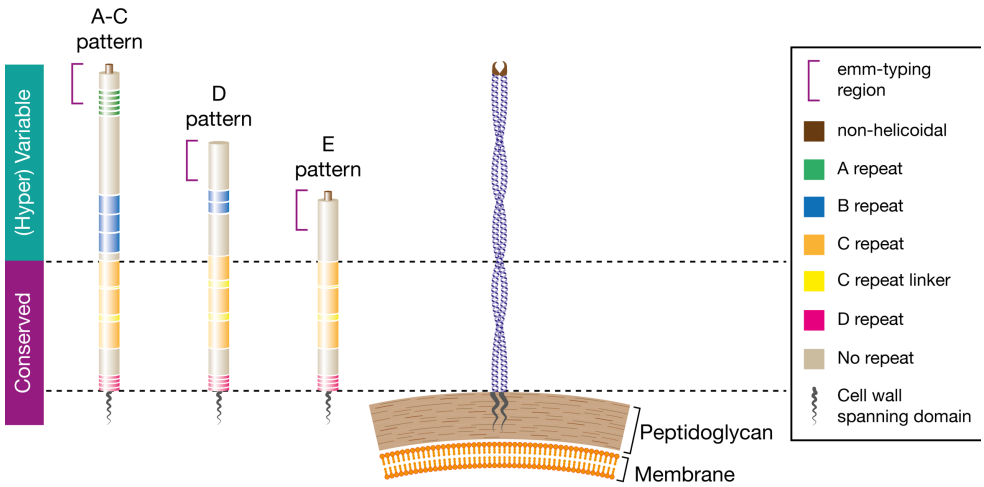


Figure 7
M proteins from various GAS strains can be classified into 3 emm patterns. Here the length of 3 representative M proteins, along with the dimensions of repeat and non-repeat sections, are depicted proportionally. A–C pattern emm-types represent the longest M proteins with roughly 230 residues in their (hyper)variable segments. In contrast, D and E patterns have (hyper)variable segments with approximately 150 and 100 residues, respectively. Most D and E pattern M proteins lack the 'A' repeats. The 'B' repeats appear in the majority of M proteins with A–C and D patterns but are mostly absent in those with E patterns. The conserved basal region contains both C and D repeats. While there are some slight differences between the C repeat segments between M proteins, the D repeats are usually highly conserved. The 'C' repeat units consist of 35 conserved residues, and these are occasionally divided by seven-residue sections known as 'C' repeat linkers. Notably, 20% of M proteins do not possess a non-helicoidal N-terminus. The hypervariable segment analyzed by the emm-typing technique is also illustrated. The M proteins M5, M80, and M77 were chosen to illustrate the structural features within each emm pattern group. Figure adapted from D. J. McMillan et al. 2013 (259).

The M protein is universally expressed by all GAS strains, however, not all strains express identical M proteins. In fact, it was the observed variability between M proteins that gave researchers the idea to use them to classify GAS strains. At first this was based on serological assays whereby immune sera were used to differentiate between M 'serotypes' (86). However, as previously mentioned, this has now been replaced by emm-typing whereby a portion of the gene encoding the M protein (emm) is assessed. This method allows for a cost-effective, rapid differentiation between distinct emm types (260). This is possible because of certain universally shared structural characteristics. The mature M protein can be roughly divided into two regions: A N-terminal (hyper)variable region ((H)VR) and a C-terminal conserved region. In this case the variability or lack thereof relates to differences between different M protein types. The N-terminal segment consists of both a

variable region, with some shared characteristics between M types, and hypervariable regions which are highly divergent in nature. The hypervariable region is found in the tip of the M protein and constitutes the region which is assessed for emm-typing (**Fig. 7**). While the variable regions of M proteins vary greatly in their N termini, they can also vary in other ways. Sequencing and assessment of the entire emm gene of hundreds of GAS isolates has shed more light on the structural characteristics that are either shared or dissimilar between M proteins. This has led to the determination of 3 distinct emm pattern groups, designated as A-C, D, and E patterns. All three groups exhibit conserved regions that include C repeats, C repeat linkers, and D repeats. The primary distinction between these pattern groups lies in their N-terminal variable regions. The A-C pattern showcases the most extended variable region. While A repeats are present in about 50% of A-C pattern M proteins, B repeats are consistently included. The variable region of the D pattern primarily includes B repeats, whereas the E pattern typically lacks any repeats. Another notable characteristic is the structure of the M protein's N-terminus which commonly loses its characteristic coiled-coil structure. Only roughly 20% of M proteins maintain a helicoidal N-terminus. Interestingly, the occurrence of this trait varies among emm pattern types. It's most prevalent in E pattern M proteins, found in 25%, while only appearing in about 10% of A-C pattern types. (**Fig. 7**) (259). Finally, it is also important to point out that roughly 75% of identified emm-types fall under the D and E pattern groups. In an epidemiological context, D and E patterns are commonly identified in situations where there are high GAS-associated mortality rates and a vast diversity of circulating emm-types. Yet, despite their significance in epidemiology, these emm-types haven't received as thorough analysis as those in the pattern A–C group. This is most likely due to the emergence of a highly virulent M1 clonal strain which is classified under the A-C group. This strain has dominated the Global North for decades and is associated with increased incidences of invasive disease (259,261).

Another important attribute of the variable N-terminal region of the M protein is the presence of imperfect repeats. These refer to the so-called heptad repeats, recurring amino acid sequences, which facilitate the formation of α-helical coiled-coil dimers (262). The conserved region typically contains regular heptad repeat patterns allowing for the formation of stable dimers at the base of the protein. In contrast, the variable regions have been shown to contain a number of imperfect heptad repeats whereby certain residues even act to destabilize the dimer. This means that certain regions of the M protein dimer are highly instable and vulnerable to environmental influences. For instance, studies reveal that the M1 protein has a propensity to unfold around temperatures of 37°C (263), which is notably aligned with the average human core body temperature (264). This means it is likely vulnerable to other factors such as organic solvents, acids, or bases. Fascinatingly, it has been shown that this instability is critical for the functionality of the M protein since idealizing mutations leading to the stabilization of the dimer also resulted in an abrogation of functionality (255,265). It's likely that this adaptability enables M

proteins with vastly different amino acid sequences to exhibit comparable functionality. For instance, while the B repeats of both M1 and M5 bind to fibrinogen, their sequences are markedly different, sharing only about 14% identity (257,266). Conversely, observations have been made where increased stability likely contributes to function. A case in point is the aforementioned M-like protein, protein H, which has been shown to bind C4BP. This protein shares many features with M protein and has been shown to dissociate into monomers at temperatures above 37°C. These monomers lack that ability to bind C4BP. Interestingly, when it binds to another human protein, IgG, its thermal stability increases. This means that protein H can maintain its dimeric structure at elevated temperatures. It's theorized that the IgG Fc binding acts to stabilize the homodimer, enabling C4BP binding even in the warmer conditions of the human circulatory system. This makes sense since this is also where the highest concentrations of complement proteins can be found (267).

The M protein stands as an example of molecular evolution's genius, showcasing versatility and adaptability in its structure and function. Its many-sided roles in GAS infections, its interactions with host components, and its potential implications in autoimmune responses illustrate its central significance in the study of this bacterium. Furthermore, the structural intricacies of the M protein, combined with its varied responses to different environmental conditions, underpin its pivotal role in GAS's survival and pathogenicity. Gaining a better understanding of the M protein not only advances our grasp on the mechanisms of action employed by GAS but also paves the way for potential therapeutic interventions, perhaps even a much-anticipated GAS vaccine.

4. Present investigation

Original Papers and Manuscripts

Paper I

High-sensitivity assessment of phagocytosis by persistent association-based normalization

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

J Immunol. 2021 Jan 1;206(1):214-224.

Paper II

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

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.

EMBO Mol Med. 2022 Dec 12;e16208.

Paper III

Group A streptococci induce stronger M protein-fibronectin interaction when specific human antibodies are bound

Sebastian Wrighton, Vibha Kumra Ahnlide, Oscar André, Wael Bahnan, and Pontus Nordenfelt.

Front Microbiol. 2023 Jan 26;14:1069789.

Paper IV

Streptococcal evasion from antibody-mediated phagocytosis is linked to fibronectin-dependent integrin modulation

Sebastian Wrighton*, Arman Izadi*, Martin Sundwall, Berit Olofsson, Vinay Swaminathan, and Pontus Nordenfelt.

* These authors contributed equally to this work

Manuscript in preparation

Paper I: High-sensitivity assessment of phagocytosis by persistent association-based normalization

Background

Phagocytosis is a vital cellular function that plays a crucial role in the immune response and overall balance within multicellular eukaryotes. It involves the engulfment of prey by cells with phagocytic abilities (59). It has been a subject of wide research interest since its discovery, and many various methods have been developed to measure it. These include direct observation through electron or light microscopy and indirect methods using markers such as radiation or fluorescence. Some approaches can distinguish between internalized and external prey through differential labeling or fluorescent changes within the phagocyte. Flow cytometry is now commonly used for high-throughput quantification (268), often with fluorescence microscopy for qualitative confirmation. Imaging flow cytometry combines both to allow detailed single-cell analysis (269).

Drawing general conclusions about phagocytosis and comparing results across different biological systems is a complex task. There are significant differences in phagocyte characteristics, such as neutrophils and macrophages, and prey properties like shape, size, surface attributes, and environmental factors (270–272). Additionally, well-known physical and experimental factors, including temperature, time, volume, and prey-to-phagocyte ratio, can affect the outcomes of phagocytosis assays. Currently, there is no standardized method to minimize the impact of experimental factors, nor a standardized definition of a phagocytic index or description of phagocytosis assays. This lack of standards creates difficulties in comparison across different experiments, systems, and laboratories, hampering reproducibility and sensitivity.

Key points and findings

- Phagocytosis is an important physiological process studied by many as a functional readout.
- Underlying factors like varying phagocytes, prey, and environments make the process highly heterogeneous.
- Difficulties in reproducibility highlight a lack of standardization in field.
- This article presents a systematic approach to normalizing factors involved in the process.
- Tools and guidelines are included to help align the method with specific research questions.

Discussion

With this article we introduce a method based on persistent association-based normalization (PAN). The theoretical foundation of this method is deeply rooted in fundamental chemistry. Specifically, it relates to a principle known as 'collision theory' which is essentially used to predict the rates of chemical reactions. Collision theory states that when reactant particles collide with the correct orientation, only a subset of these collisions leads to a perceptible change; these successful interactions are termed successful collisions (273). Importantly, the number of successful collisions can be altered by manipulating certain factors. These include the concentration of the reactants as well as the temperature and pressure at which the interactions take place. Furthermore, catalysts can be used to speed up the reaction and change the mechanism by which molecules collide. Similarly, phagocytosis can be viewed as interactions between reactants, whereby instead of molecules the reactants are phagocytes and prey. During the process of phagocytosis, phagocytes and prey will interact with each other. However, as in collision theory, only some of those interactions will lead to recognition and binding to the prey or 'persistent association'. Just as in collision theory, the process of phagocytosis, which begins with persistent association, can be manipulated by varying certain experimental parameters. Some of these are inversely correlated with phagocytosis like volume and the concentration of phagocytes. However, others like the concentration of prey, the incubation time, and the incubation temperature are directly correlated. Intriguingly there are even parallels to the concept of a catalyst since depending on the phagocyte of interest, the addition of molecules such as antibodies or complement factors can increase the efficiency of the phagocytic reaction. These many parallels highlight the fact that, while biology is full of randomness, there are many factors that can be normalized and accounted for. By doing so it is possible to minimize the inevitable experimental noise.

The actual execution of the PAN method is founded in dose-response curve analysis. This form of analysis is commonly used in pharmacology where it is used to test the pharmacological effect of certain drugs. There the concept of half-maximal effective concentration (EC_{50}) is used to assess the pharmacological potency of a substance. Specifically, the EC_{50} indicates the concentration of a drug required to achieve a response midway between the baseline and the maximum possible effect. In PAN a MOP_{50} value is determined, whereby this denotes the number of preys per phagocyte (MOP) that are needed to achieve 50% persistent association. The MOP_{50} value is determined by running MOP curves whereby persistent association is assessed at varying MOPs. These results can then be assessed by non-linear regression analysis, yielding a MOP_{50} value. Importantly, these MOP curves are powerful analytical tools in their own right. They can reflect the respective effectiveness of a phagocytic reaction and furthermore offer insights into a system's dynamic range. These curves can pinpoint the specific MOP levels where more in-depth analyses like microscopy should be conducted. This enhances the assay's sensitivity and could also cut down

on time spent setting up unnecessary experiments. Finally, it should be mentioned that the PAN method is fully compatible with microscopy-based phagocytosis assays. However, this would most likely result in a significantly slower throughput compared to flow cytometry-based techniques.

Overall, the PAN method has been critical for my journey as a PhD student. Because of my background in medicine, I initially had very little formal experience in the lab. When planning my first experiments, I often felt overwhelmed by all of the variables I had to account for. Starting to use PAN was a definitive turning point. Its theoretical foundation was thought provoking and gave me novel perspectives on biology and research in general. While before my focus would solely be on the condition or therapy I was interested in testing, now I began seeing experiments more as chemical reactions with many variables simultaneously affecting the outcomes. In this way it has been critical in the planning and execution of my experiments – many of which are included in this thesis.

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

Background

Antibodies, essential components of the immune system, neutralize, and opsonize external pathogens such as bacteria and are produced by B cells (274). These Abs are created through processes like V(D)J recombination and somatic hypermutation, resulting in a diverse repertoire of Ab variable domains (275,276). IgG Abs typically bind to an antigen through one of their Fab domains. They can also bind to an object with both Fab domains simultaneously when two target antigens lie within close proximity of each other. This dual-Fab trans-binding can increase the binding strength through avidity, a process that depends on antigen density and organization (277). Once bound, IgG can trigger the clustering of Fc receptors on immune cells, inducing effects such as phagocytosis and immune activation.

Group A streptococcus (GAS) is a common human pathogen causing significant morbidity and mortality worldwide. GAS has evolved multiple mechanisms to counter the human immune response, including resistance to phagocytosis and targeting immunoglobulins through specific proteins. A notable protein, M protein, contributes to phagocytosis resistance, induces vascular leakage, and can reduce phagocytosis by capturing IgG Fc domains. Despite the humoral immune response to GAS infections, repeated exposures are needed to generate protective memory B cell immunity (278). M protein constitutes one of the prime immunizing Ags but no effective vaccine has been approved (279). The generation of mAbs targeting bacteria has become a topic of increasing interest within the field of infectious diseases. In fact, many mAb therapies targeting various bacterial antigens are

currently in development (280). While high production costs and prices makes such therapies ineffective from a public health standpoint, they could certainly be useful in the treatment of rare invasive GAS disease manifestations. Moreover, such monoclonals can act as instrumental tools in fundamental biological research.

Key findings

- Monoclonal antibodies were generated targeting the central region of GAS M1 protein.
- Dual-Fab cis-binding is novel form of Ab binding where both Fabs bind to distinct epitopes on one M protein molecule.
- The dual-Fab cis-binding Ab (Ab25) was shown to trigger immune effector functions while regular binding Abs targeting the same region did not.
- Ab25 protected mice in a GAS infection model.
- Ab25 was shown to cross-react with a number of different M types.

Discussion

This research article introduces a mAb (Ab25) that binds to the M protein's central region. Unlike typical Abs, Ab25 attaches to the M protein at two distinct epitopes simultaneously through a novel phenomenon known as dual-Fab cis-binding (DFCB). It represents a bivalent interaction spanning the M1 protein's S region, confirmed through three independent methods. The research demonstrates that DFCB can potentially enhance Ab function and offers insight into effective opsonophagocytic interactions. However, it remains to be seen what role DFCB Abs play in the broader immune response. The binding properties exhibited by Ab25 bring to mind the mAb 2G12. This human mAb targeting the HIV glycoprotein gp120 was discovered in 1996 and was also observed to exhibit unique binding characteristics (281). Similarly, to Ab25, it bound gp120 with both its Fabs simultaneously, however, upon closer observation its binding characteristics were very different. In the case of 2G12, the DFCB was occurring due to a mutation in the mAb's hinge region. This mutation led to the dimerization of both Fabs, resulting in one large Fab that could bind to gp120. This is where the binding characteristics of the two mAbs differ fundamentally since Ab25 was shown to bind two distinct, spatially separated epitopes. Moreover, 2G12's unique binding characteristics were not found to influence the mAb's immune function.

Another remaining question concerns the broader significance of the DFCB. We have so far found one such mAb, however, our results comparing single Fabs with $F(ab')_2$ fragments from both pooled and convalescent plasma showed that Fabs derived from convalescent plasma bound less to GAS than $F(ab')_2$ fragments. This was not seen with the pooled Abs which could indicate that previous infection can lead to the preferential development of DFCB Abs and that these make up a

substantial portion of the Abs targeting GAS. However, we must keep in mind that this relies on the assumption that all the DFCB Abs can only bind in this manner. By this, I mean that, as with Ab25, these Abs can only bind with both their Fabs simultaneously since individually they lack the affinity to bind to their respective epitopes. In any case, more DFCB mAbs, both targeting GAS and other antigens will need to be identified in order to make wider conclusions regarding this phenomenon.

In order for M protein bound IgG to trigger effector functions, an immune cell's FcγR must interact with its Fc domain. Antibodies are highly flexible proteins (282), thus being bound by a single Fab offers a significant freedom of movement. This means that the Fc domain could be pointing anywhere from downwards towards the bacterial surface to upwards towards the M proteins N-terminus. DFCB entails the simultaneous binding of both Fabs reducing the degree of spatial freedom of the Fc domain. The Fc domain orientation of a DFCB Ab would preferentially be perpendicular in relation to the M protein. Interestingly this happens to be the optimal angle for FcγR-Fc interaction (283). Moreover, it was shown that since Ab25 binds above and below the M protein's Fc binding domain it was able to reduce the M protein's ability to bind non-specific Abs via their Fcs. While it is not fully understood how this mechanism benefits GAS, it is possible that this could lead to further protection for the host. Overall, this study offers insight into immune responses against GAS and reveals the potential importance of DFCB Abs. These findings could be helpful in the future development of monoclonal Ab therapies or vaccines.

Paper III: Group A streptococci induce stronger M protein-fibronectin interaction when specific human antibodies are bound

Background

A key aspect of GAS biology is its relationship with Fn, a high-molecular-weight glycoprotein found in both soluble and insoluble forms. Within the host, Fn acts as an adaptor protein of the ECM, facilitating cell interactions with their environment. GAS has been shown to bind Fn, enhancing its adhesion and invasion of host cells. This binding is mediated by surface-expressed bacterial proteins possessing Fn-specific binding domains. These include specialized Fn binding proteins, such as PrtF1/SfB1, PrtF2, FbaA, and others. Some FnBPs have been found to promote epithelial cell invasion by collaborating with integrins on epithelial cells (284,285). Around 12 GAS surface proteins facilitate Fn binding, indicative of an evolutionarily driven process.

The GAS M protein illustrates the bacterium's evolutionary manipulation of the host immune system. With a highly variable N-terminal domain, the M protein is the primary target of the humoral immune response. Some Abs target the conserved C

region, serving as cross-serotype antigens although these have been associated with a lack of immunological function (286). Certain M types have been found to bind Fn, particularly the M1 protein.

Key findings

- Polyclonal plasma antibodies from patients who have recently recovered from a GAS infection increase Fn binding to GAS.
- Monoclonals targeting the central region of the M protein can also increase Fn binding.
- Fn binding occurs in the N terminal region of the M1 protein.
- Crosslinked Abs and Abs lacking Fc domains do not affect Fn binding to M protein.
- Ab-mediated Fn binding reduces opsonophagocytosis.
- Ab-mediated Fn binding occurs in 3 of 5 tested emm-type strains and is not linked to sequence homology.

Discussion

In this study we initially began studying the M1 GAS strain SF370. Since it is known that the M1 protein can bind Fn we were curious to see the effects of Abs on its binding capacity. During initial testing we were surprised to find that firstly, GAS incubated in purified Fn was hardly binding any Fn and secondly, that when we incubated GAS in plasma from donors who had recently recovered from a GAS infection, Fn binding shot up. At first, we were worried we had mixed up the samples since we were expecting that post-infection samples with high titers of anti-GAS Abs would neutralize any binding sites on the M protein; not have the opposite effect. Additionally, we were confused by how little Fn the 'Ab untreated' GAS was binding. After all, it's not only M1 that should be binding Fn; SF370 has been shown to have another Fn binding protein, FbaA (287). However, as strange as is seemed it turned out to be true. At this point we didn't know if it was Abs or something else in the convalescent plasma that was having this effect, so we decided to test our anti-M monoclonals. Interestingly, we found that 2 of 3 monoclonals targeting the M protein's central region, also led to this increase in Fn binding.

While we explored several avenues in this article, much is still unknown about this phenomenon. For example, it's still unclear why SF370 and other M1 strains we have tested bind so little Fn. This became even more apparent when testing the other emm-type strains since they all bound Fn very efficiently. In the discussion section of the article, we suggest that M1 could be taking over the 'work' of Fn binding and that this has led to a reduced expression of FbaA. While this is an intriguing hypothesis, it is important to consider other possible explanations. First it would be important to test if these strains do in fact express FbaA. Then it would also be

critical to test more recent clinical isolates since it is common for laboratory strains to lose pathophysiological characteristics (288). Another aspect that deserves further inspection is the biological relevance of this mechanism. While we have shown that it can reduce opsonophagocytosis it is hard to dismiss a feeling that there must be more to this phenomenon. The most obvious question that should be explored next is: does it affect adhesion? Considering the 2-step adhesion proposed by R. J. Doyle and colleagues (224) it could make a lot of sense that M1 binds Fn so weakly. This model proposes that LTA serves as a low affinity adhesin that allows GAS to be drawn closer to the host epithelium. This is followed by a second step entailing multiple high affinity binding interactions which makes the adhesion essentially irreversible. It is possible that, in an environment that has low concentrations of both IgG and Fn, like the nasopharynx, M protein first functions as a low affinity adhesin. In this way it could help GAS to get an initial grip on Fn that is found embedded within the mucus layer. However, in an environment with high concentrations of IgG, such as a wound, the M1 protein can immediately be repurposed as a second step adhesin since the binding Abs enhance its Fn binding affinity.

This would be yet another example of the M protein's extraordinary ability to adapt to its environment; constituting a fascinating protein which shines even more brightly because of its imperfections. Unlike many other proteins, it is not its structural integrity which allows it to function, but rather it is the opposite. The destabilizing residues found within this coiled-coil protein's structure make it inherently instable. This means that it readily unfolds at temperatures above 37°C (289). It is no coincidence that the M protein is primarily found within the human body, an environment that constantly keeps it teetering on the edge of unraveling. It is here that the M protein truly begins to excel. Its instability gives it the ability to bind to many diverse ligands and essentially 'react' to its environment. Considering this, perhaps it makes sense that this protein would also actively seek to draw the attention of the adaptive immune system so that it could use the ensuing Abs to alter its own function.

Paper IV: Streptococcal evasion from antibody-mediated phagocytosis is linked to fibronectin-dependent integrin modulation

Background

GAS is renowned for possessing the ability to survive in human blood (290); an impressive feat since this constitutes an harsh environment that not many pathogens can withstand. It is one laden with destructive immune cells, Abs, antimicrobial peptides, clotting factors, and complement. However, it is important to keep in mind that the mucosal niche of the nasopharynx is where long-term asymptomatic carriage occurs. This mode of interaction benefits GAS by allowing prolonged

replication and enhancing horizontal transmission, particularly within close-knit communities like schools and families (215). A feature that clearly differentiates these two host niches is the concentration of soluble proteins, including the concentration of soluble Fn. While blood contains Fn concentrations between 300-400 µg/ml, saliva only contains around 0.05-0.15 µg/ml (291,292).

Antibodies play an important role in the host defence against GAS (293). This is further evinced by the fact that GAS has developed numerous virulence factors specifically targeting Abs – specifically IgG (153,240,241,246). IgG is found in 4 distinct subclasses whereby each is associated with specific FcγR affinities and effector functions (37). It has been found that the IgG3 subclass acts as a particularly potent opsonin and is associated with GAS infections (43,155).

FcγR-mediated phagocytosis is a process characterized by intracellular actin remodeling leading to the formation of a phagocytic cup (76). During the course of this process, membrane protrusions known as pseudopods progressively wrap around the target prey until it is finally engulfed (294). There are a number of different FcγRs which are associated with distinct immune effector functions. These are expressed by different cell lines and can bind IgG subclasses with varying affinity (43). In the previous study the monocytic cell line THP-1 was used to study the effects of Ab-enhanced Fn binding on phagocytosis. These are known to constitutively express FcγRI and FcγRII which can both induce phagocytosis (295). In this study we furthermore assess neutrophils which are the most efficient phagocytes. These constitutively express FcγRIIa, as well as FcγRIIIb and FcγRIV, whereas the expression of FcγRI must be induced by stimulation with G-CSF (60).

Key findings

- All IgG3 subclasses can enhance M protein Fn binding.
- Both high and low Fn concentrations can lead to a reduction in opsonophagocytosis.
- Neutrophil-mediated phagocytosis is also affected by Ab-mediated Fn binding.
- Incubation in saliva reduces the opsonic efficiency of Abs that mediate Fn binding.
- The reduction in opsonophagocytosis is not due to inhibited Fc-FcγR engagement.
- Reduction in opsonophagocytosis is likely linked to integrin modulation.

Discussion

In this final paper we revisit the phenomenon of Ab-enhanced Fn binding. Since we knew that the IgG1 Fc domains were a prerequisite, we were curious to assess the effects of other IgG subclasses. The results showed that while all subclass variants led to an increase in Fn binding, they did not do so to the same degree. However, this most likely has to do with the respective Abs binding affinities rather than the interaction between IgG Fc domains and Fn.

Considering that Fn is found in such different concentrations throughout the body, specifically in the two main niches that GAS inhabits, we wanted to test the effects of similar Fn concentrations of phagocytosis and do so with a more physiologically relevant model phagocyte – neutrophils. Firstly, we found that, just like with the monocytic cell line THP-1, Ab-enhanced Fn binding led to a reduction in phagocytosis. This was not seen when using pooled intravenous IgG (IVIG) which was previously shown to have little effect on Fn binding. Secondly, we could show that low (1µg/ml) and high (400µg/ml) concentrations of Fn could lead to an inhibition in phagocytosis. These results were highlighted further when we could show that GAS incubated in varying concentrations of saliva could lead to a reduction in phagocytosis by THP-1 cells. While saliva had little effect on IVIG opsonized GAS, there was a striking effect on GAS opsonized with Ab25. Here we could clearly see that increasing saliva concentrations indirectly correlateded with phagocytic efficiency. These results offer further evidence that this mechanism is adapted to the mucosal niche, allowing GAS to inhibit the effect of dangerous opsonic Abs. However, these results should be interpreted with caution. Firstly, this experiment was done with a cell line and would need to be replicated with primary phagocytes. Moreover, we cannot rule out that other factors found in saliva were contributing to the effect. Further experiments are needed to validate these results and confirm that Fn is indeed the causative agent.

Finally, we wanted to better understand the mechanism behind this decrease in opsonophagocytosis. We first began by testing whether GAS bound Fn could interfere with the interaction between IgG and FcγRs. We found no differences concerning GAS interaction with any of the F FcγRs, regardless of the Ab and Fn treatment. While this result would need to be confirmed through other means, it is likely that Fn is not leading to a reduction in FcγR interaction. We decided to, next, investigate the role of integrins. On the one hand, we did so because Fn is classically known for its interaction with integrins. On the other hand, it had just recently been established that a certain promiscuous integrin, αMβ2 (CR3), is involved in the process of FcγR-mediated phagocytosis. A study found that phagocytes lacking αMβ2 were slower at engulfing prey and formed irregular, elongated phagocytic cups (76). This made sense since we had not seen a total abolishment of phagocytosis, but rather it seemed like a reduction in efficiency. To test whether integrins are involved in the process we repeated a previous experiment, but this time included the integrin activating agent Manganese (Mn^{2+}). While it didn't lead

to a consistent reduction in phagocytosis, we saw that, specifically for Ab25 treated GAS, it led to significantly increased variability between experiments. The fact that we saw fluctuations between sharp increases and decreases in phagocytosis reminds of the biphasic manner in which integrins bind to Fn within the ECM (296). It is possible that by binding Fn, GAS is able to interfere with the cellular mechanosensing apparatus, resulting in a reduction in opsonophagocytic killing. In fact, this is not the first time that interactions between β2 integrins and M protein-host protein complexes have been described. A 2004 research article showed that neutrophils recognize M protein/Fibrin complexes through β2 integrins. This was shown to lead to neutrophil activation and the release of the inflammatory mediator heparin binding protein (258).

Concluding remarks and future perspectives

Concluding Remarks

The body of work presented in this thesis, seeks to shed light on the complex interactions between mAbs and the GAS M protein. It does so by combining novel in-depth analytical methodologies with experimental studies. The first paper, "High-sensitivity assessment of phagocytosis by persistent association-based normalization," describes a reliable method to measure phagocytosis. The method's adaptability and precision have subsequently driven the research for the other papers in this collection. The next three papers delve into the specifics of how human mAbs interact with M protein and the functional outcomes of these interactions. These include a novel bivalent Ab binding mode, the enhancement of M protein's affinity for Fn when specific human Abs are present, and the mechanisms of GAS immune evasion tactics likely involving Fn-dependent integrin modulation. Each of the three papers highlight the remarkable extent to which GAS has fine-tuned its relationship with its human host, casting the M protein as a pivotal figure in this dynamic. Initially, it appeared that the immune system had gained a tactical advantage through Ab25, an Ab exhibiting a unique binding profile capable of bypassing the M protein's anti-phagocytic properties. Yet, GAS was already ahead of the curve. The same Ab that initially seemed promising also enhanced the M protein's affinity for Fn. This enhanced binding to Fn led to a reduction in the efficiency of opsonophagocytosis, thereby potentially diminishing the Ab's *in vivo* efficacy. These results serve as a compelling testament to the resilience and adaptability of GAS and underscore the formidable challenges we face in designing effective therapeutic interventions against this elusive pathogen.

Future Perspectives

When I started my PhD, I was filled with a sense of endless time and possibility for exploring diverse research topics. However, I quickly realized that scientific discovery is rarely straightforward. Despite the numerous obstacles – some more significant than others – that initially caused me great stress, I've come to appreciate

the valuable lessons and problem-solving skills they have imparted. As my time as a PhD student draws to a close, I find myself astonished at how quickly this time has passed. This becomes particularly apparent considering the abundance of research questions that still await exploration. Here I will shortly discuss some aspects of these projects that could warrant further exploration:

As a starting point, I believe the phenomenon of DFCB Abs should be further explored. Is it a general phenomenon that convalescence leads to the generation of more DFCB Abs? If so, is this restricted to Abs targeting GAS or perhaps only M protein? It is possible that the unique 3-dimensional structure of M protein allows for this form of binding. However, there are many bacterial and even host proteins that share many of its structural characteristics. Regardless, a first crucial step will entail the identification of novel DFCB Abs. Careful assessment of the binding characteristics of such Abs could offer insight into the prerequisites for, as well as benefits of DFCB.

Further, the ability of some Abs to enhance the binding of Fn to M protein leaves much to explore. Firstly, we have seen that 2 of 4 tested anti-GAS mAbs are able to enhance Fn binding. It would be important to screen more such mAbs to test how generalizable this phenomenon is. Moreover, it is unclear why these kinds of Abs seem to become enriched after recent infection with GAS. Since essentially everyone has at some point recovered from a GAS infection it would be interesting to explore why pooled Abs from healthy donors no longer show this phenotype. Are these kinds of Abs recognized by the immune system over time and in some way downregulated? To better understand the mechanistic aspects behind Ab-enhanced Fn binding to M protein it will be interesting to compare Ab25 and Ab26MS since they are both opsonic but only one of them enhanced Fn binding. This most likely has to do with the specific binding site on M rather than structural differences between the two Abs. This seems unlikely because they both share identical constant domains. However, since we found that the IgG Fc domain was necessary for Fn binding to occur, it could be interesting to assess the importance of certain regions within the IgG Fc. This could help to understand if the Fc is directly interacting with Fn.

Another aspect of Ab-enhanced Fn binding which remains to be explored is the impact of soluble M protein. During infection with GAS, M protein is enzymatically cleaved by both bacterial as well as host proteases. This results in the release of soluble M protein which has many times over been shown to lead to a number of detrimental effects for the host (238,258). We have not yet assessed whether Ab-enhanced Fn binding can also occur in solution. If so, this may have far reaching implications. For example, many receptors expressed on the surface of host cells contain immunoglobulin-like domains and/or FNIII domains. One example is the neural cell adhesion molecule (NCAM) which contains both (297). Although NCAM is often primarily associated with cells of the central nervous system it can also be found on various lymphoid cells including T cells, Natural killer (NK) cells

cells, and dendritic cells (298). In fact, recent studies have found that NCAM may even serve as a form of PRR on NK cells, helping to control infection with the fungal pathogen *Aspergillus fumigatus*. Interestingly the researchers found that blocking NCAM with a mAb led to reduced fungus-mediated NK cell activation and reduced cytokine secretion (299). Since the spatial arrangement of both the two FNIII and five immunoglobulin-like domains within NCAM seem to match the binding site arrangement on the M1 protein (300,301), it is plausible that soluble M1 protein could act similarly to a blocking mAb – impairing the function of certain immune cells. This could constitute yet another elaborate immune evasion mechanism employed by GAS. As a matter of fact, binding of M protein to NCAM has already been described (302), however, it seems this was never further investigated. While this hypothesis may seem somewhat far-fetched, I believe that it could warrant future exploration.

Papers not included in this thesis

Spike-dependent opsonization indicates both dose-dependent inhibition of phagocytosis and that non-neutralizing antibodies can confer protection to SARS-CoV-2.

Wael Bahnan, **Sebastian Wrighton**, Martin Sundwall, Anna Bläckberg, Olivia Larsson, Urban Höglund, Hamed Khakzad, Magdalena Godzwon, Maria Walle, Elisabeth Elder, Anna Söderlund Strand, Lotta Happonen, Oscar André, Johannes Kumra Ahnlide, Thomas Hellmark, Vidar Wendel-Hansen, Robert Pa Wallin, Johan Malmstöm, Lars Malmström, Mats Ohlin, Magnus Rasmussen, Pontus Nordenfelt.

Front Immunol. 2022 Jan 14;12:808932.

Nanoscale binding site localization by molecular distance estimation on native cell surfaces using topological image averaging.

Vibha Kumra Ahnlide, Johannes Kumra Ahnlide, **Sebastian Wrighton**, Jason P Beech, Pontus Nordenfelt.

Elife. 2022 Feb 24;11:e64709.

Subclass-switched anti-Spike IgG3 oligoclonal cocktails strongly enhance Fc-mediated opsonization

Arman Izadi, Arsema Hailu, Magdalena Godzwon, **Sebastian Wrighton**, Berit Olofsson, Tobias Schmidt, Anna Söderlund-Strand, Elizabeth Elder, Sofia Appelberg, Maria Valsjö, Olivia Larsson, Vidar Wendel-Hansen, Mats Ohlin, Wael Bahnan, Pontus Nordenfelt.

Proc National Acad Sci. 2023 120;e2217590120.

The impact of antibody hinge flexibility in Fc-mediated protection against streptococci

Arman Izadi, Yasaman Karami, Eleni Bratanis, **Sebastian Wrighton**, Hamed Khakzad, Maria Nyblom, Berit Olofsson, Lotta Happonen, Di Tang, Michael Nilges, Johan Malmström, Wael Bahnan, Oonagh Shannon, Lars Malmström, and Pontus Nordenfelt.

Currently in revision.

IdeS, a secreted proteinase of Streptococcus pyogenes, is bound to a nuclease at the bacterial surface where it inactivates opsonizing IgG antibodies.

Inga-Maria Frick, Lotta Happonen, **Sebastian Wrighton**, Pontus Nordenfelt, and Lars Björck.

Currently in revision.

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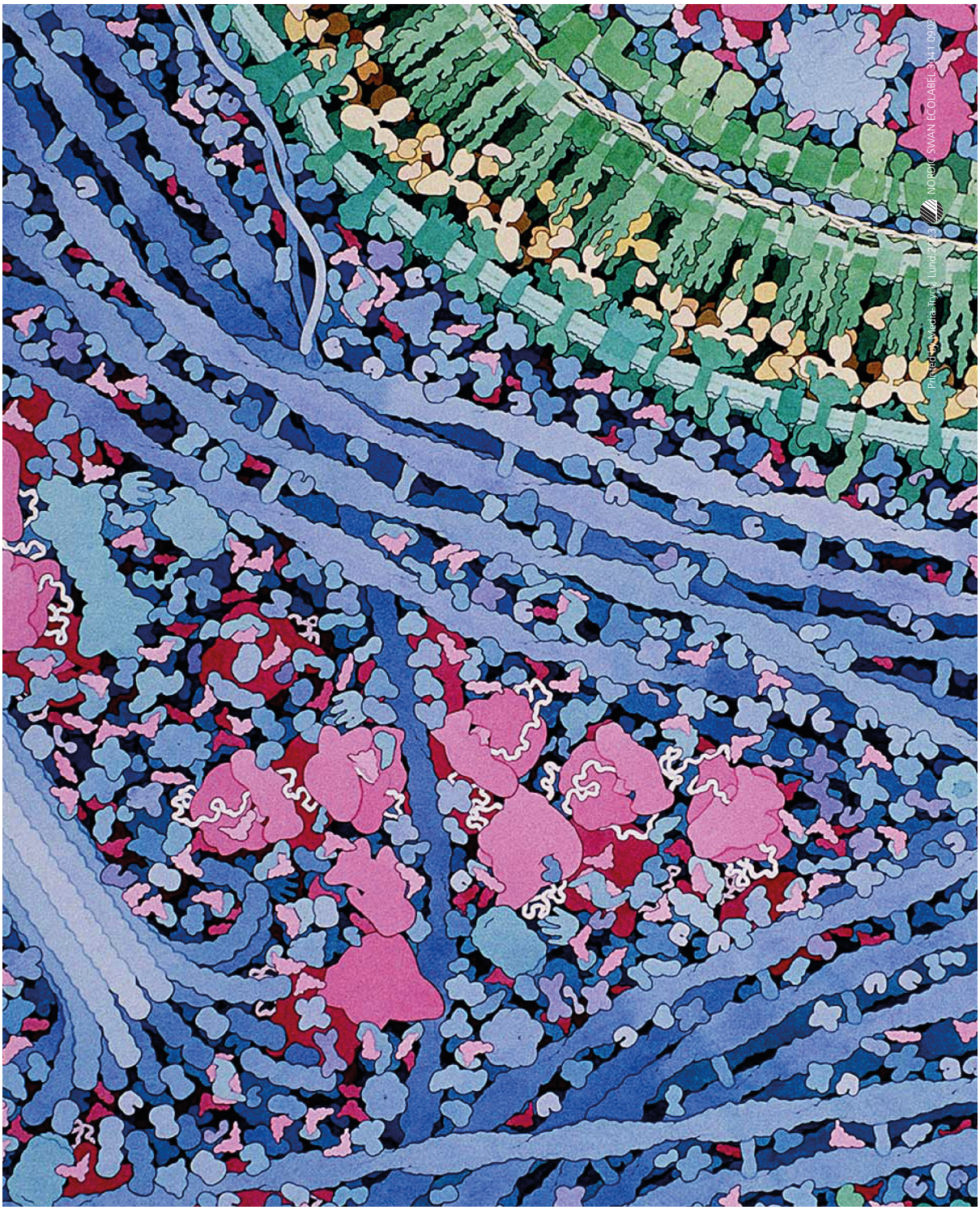
Appendix (Papers I-IV)



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