Fc-Engineered Antibodies Against SARS-CoV-2 and Streptococcus pyogenes: Therapeutic Potential via Enhanced Opsonization

Izadi, Arman

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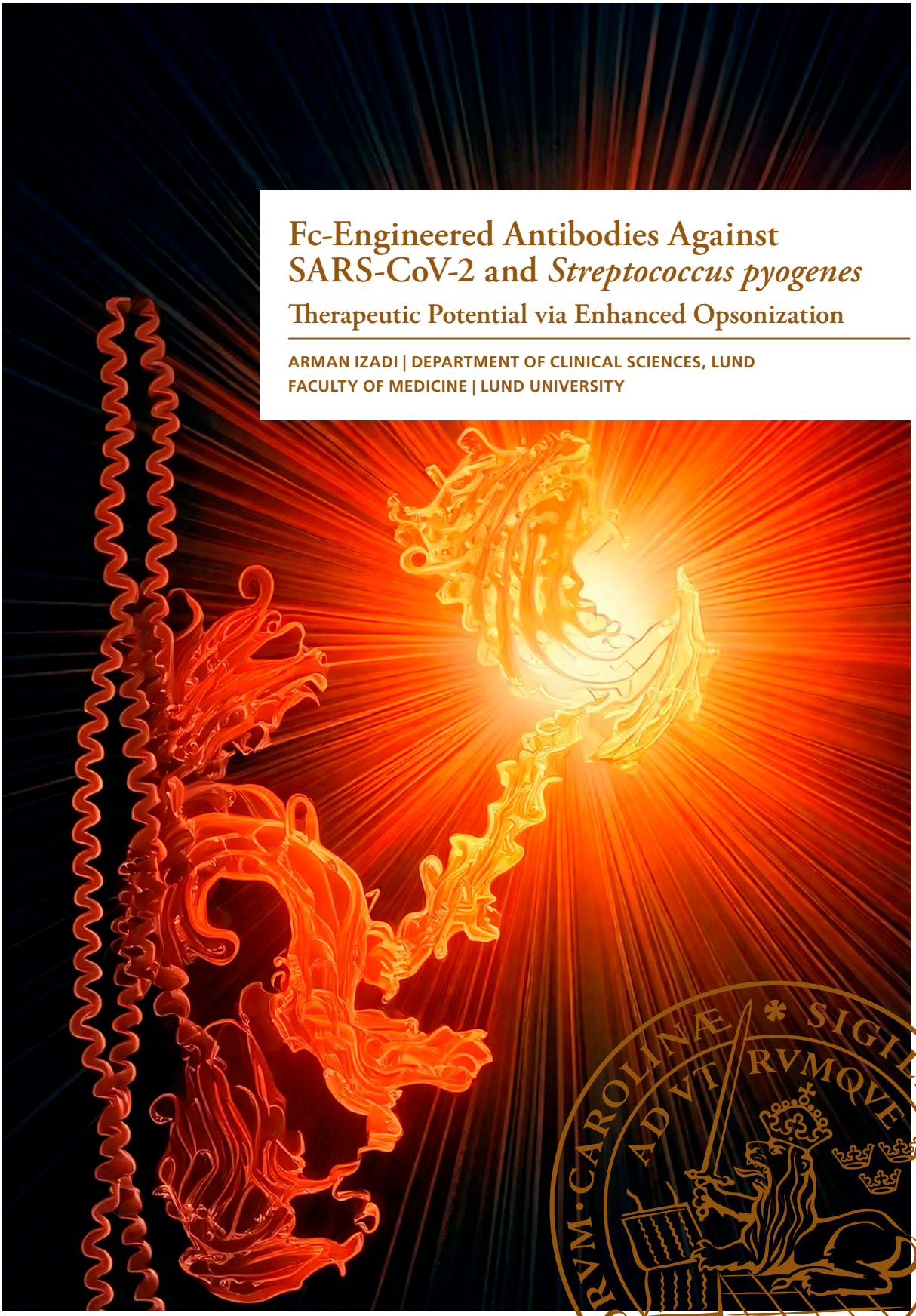
Therapeutic Potential via Enhanced Opsonization

ARMAN IZADI | DEPARTMENT OF CLINICAL SCIENCES, LUND
FACULTY OF MEDICINE | LUND UNIVERSITY



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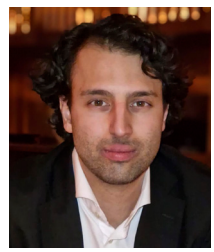

Arman is a medical doctor with a strong interest in infectious diseases and immunology. He earned his MD from Lund University and is currently completing his clinical training at Karolinska University Hospital. He believes in the synergy between bedside clinical experience and wet-lab research as a powerful way to solve real-world medical problems. His research reflects a broader ambition to help patients through translational science, with a particular focus on monoclonal antibodies as a promising approach for treating infectious diseases such as severe *Streptococcus pyogenes* infections.

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Fc-Engineered Antibodies Against SARS-CoV-2 and *Streptococcus pyogenes*

Therapeutic Potential via Enhanced Opsonization

Arman Izadi

LUNDS UNIVERSITET

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 12th of June at 9.00 in Belfragesalen, Biomedical Center (BMC), Lund, Sweden

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Monoclonal antibody (mAb) development has progressed in many fields, especially oncology, but remains limited in infectious diseases. Life-threatening infections like SARS-CoV-2 and invasive *Streptococcus pyogenes* (e.g., necrotizing soft tissue infections with over 20% mortality) could benefit from mAb therapy. Most SARS-CoV-2 antibodies are expressed as the IgG1 subclass, focusing on neutralizing the virus, with little focus on enhancing immune system interaction. Only one protective monoclonal antibody has been described for *Streptococcus pyogenes* (group A streptococcus, GAS). This thesis explores improving the therapeutic potential of mAbs against both types of pathogens through Fc-engineering. In **Paper I**, we found that non-neutralizing antibodies protect mice from a lethal infection with the original Wuhan strain of SARS-CoV-2. While neutralizing RBD antibodies have been shown to protect animals, this is the first report of a non-neutralizing RBD antibody providing protection. The mechanism was via Fc-mediated functions like phagocytosis, which we could enhance further by switching the constant domain from IgG1 to IgG3. A cocktail of IgG3 antibodies produced the strongest response. We also discovered that the constant domain affects antigen binding, challenging the belief that the variable and constant domains are independent. Due to mutations in the SARS-CoV-2 spike protein, most neutralizing antibodies become ineffective. In **Paper III**, we show that one of our non-neutralizing antibodies remains functional and binds after 4 years of mutations, making it potentially a more durable therapeutic option than neutralizing antibodies. The non-neutralizing antibody improved clinical symptoms in mice infected with the highly mutated JN.1 variant of SARS-CoV-2, which showed surprisingly low virulence in this model – providing insight into the viral evolution. **In Paper II,** we generated all four human subclasses of the protective anti-M monoclonal antibody against *Streptococcus pyogenes*. We found that the IgG3 version had reduced binding to the antigen, but surprisingly exhibited much higher opsonophagocytic activity than IgG1. This was likely due to its longer hinge domain, which provided greater flexibility, as supported by molecular dynamics simulations. These simulations showed differences in hydrogen bond and salt-bridge interactions between the M protein and the IgG1 and IgG3 subclasses, potentially explaining the reduced binding of IgG3. We further demonstrated the importance of the hinge domain by creating IgG1-IgG3 hybrid subclasses. The $IgGh_{47}$ version, with a 47 amino acid IgG3 hinge, showed an even stronger opsonic function than both IgG1 and IgG3. In a mouse model of severe GAS infection, only IgGh47 protected mice, while the natural subclasses did not. This enhanced function was transferable to different clinical isolates and even SARS-CoV-2 spike antibodies. In **Paper IV**, $IgGh_{47}$ versions of anti-spike antibodies improved phagocytosis against mutated SARS-CoV-2 variants and other Betacoronaviruses, like SARS-CoV and MERS-CoV. These antibodies also showed unexpectedly high affinity for Fc receptors, unlike their IgG1 parents. Overall, this thesis shows that the $IgGh_{47}$ subclass is a promising backbone for enhancing non-neutralizing protective functions against *Streptococcus pyogenes*, SARS-CoV-2, and potentially other Betacoronaviruses. Additionally, the altered antigen-binding observed in **Papers I-II**, along with the molecular dynamics simulations, challenges the traditional view of antibody variable and constant domain independence.

Key words: Monoclonal antibodies, IgG subclasses, Hinge, Fc-function, phagocytosis, SARS-CoV-2, Betacoronaviruses, *Streptococcus pyogenes*

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Arman Izadi

LUNDS
UNIVERSITET

Arman Izadi

Department of Clinical Sciences, Lund Division of Infection Medicine Faculty of Medicine
Lund University
Biomedical Centre, D14
221 84 Lund, Sweden

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Faculty of Medicine
Department of Clinical Sciences, Lund

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Dedicated to my family

"Let yourself be silently drawn by the strange pull of what you really love. It will not lead you astray" –
Rumi, a 13th century Persian poet

TABLE OF CONTENTS

ABSTRACT

Monoclonal antibody (mAb) development has progressed in many fields, especially oncology, but remains limited in infectious diseases. Life-threatening infections like SARS-CoV-2 and invasive *Streptococcus pyogenes* (e.g., necrotizing soft tissue infections with over 20% mortality) could benefit from mAb therapy. Most SARS-CoV-2 antibodies are expressed as the IgG1 subclass, focusing on neutralizing the virus, with little focus on enhancing immune system interaction. Only one protective monoclonal antibody has been described for *Streptococcus pyogenes* (group A streptococcus, GAS). This thesis explores improving the therapeutic potential of mAbs against both types of pathogens through Fc-engineering. In **Paper I**, we found that non-neutralizing antibodies protect mice from a lethal infection with the original Wuhan strain of SARS-CoV-2. While neutralizing RBD antibodies have been shown to protect animals, this is the first report of a non-neutralizing RBD antibody providing protection. The mechanism was via Fc-mediated functions like phagocytosis, which we could enhance further by switching the constant domain from IgG1 to IgG3. A cocktail of IgG3 antibodies produced the strongest response. We also discovered that the constant domain affects antigen binding, challenging the belief that the variable and constant domains are independent. Due to mutations in the SARS-CoV-2 spike protein, most neutralizing antibodies become ineffective. In **Paper III**, we show that one of our non-neutralizing antibodies remains functional and binds after 4 years of mutations, making it potentially a more durable therapeutic option than neutralizing antibodies. The non-neutralizing antibody improved clinical symptoms in mice infected with the highly mutated JN.1 variant of SARS-CoV-2, which showed surprisingly low virulence in this model – providing insight into the viral evolution. **In Paper II,** we generated all four human subclasses of the protective anti-M monoclonal antibody against *Streptococcus pyogenes*. We found that the IgG3 version had reduced binding to the antigen, but surprisingly exhibited much higher opsonophagocytic activity than IgG1. This was likely due to its longer hinge domain, which provided greater flexibility, as supported by molecular dynamics simulations. These simulations showed differences in hydrogen bond and salt-bridge interactions between the M protein and the IgG1 and IgG3 subclasses, potentially explaining the reduced binding of IgG3. We further demonstrated the importance of the hinge domain by creating IgG1-IgG3 hybrid subclasses. The $IgGh_{47}$ version, with a 47 amino acid IgG3 hinge, showed an even stronger opsonic function than both IgG1 and IgG3. In a mouse model of severe GAS infection, only IgGh47 protected mice, while the natural subclasses did not. This enhanced function was transferable to different clinical

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PREFACE

There is a common saying that only two people read your thesis: your supervisor and your opponent. That is fine by me because I wrote this thesis as if I were handing it to my younger self, someone who enjoyed diving into extensive details to understand the big picture (often ending up with even more questions than before). Writing with this perspective made the process an excellent learning experience, allowing me to explore subjects beyond what I encountered during my MD and PhD studies. Balancing writing the thesis with my work-schedule as an MD was a true test of resilience and mental endurance. The PhD journey is a unique experience both challenging and rewarding and I hope that resilience is one of the most valuable skills I take away from it.

A few words on the structure of this thesis: It begins with an overview of the immune system (Chapter 1), progressively increasing in detail to provide context for the more specific research chapters that follow. My goal was to explore the immune system's intricacies, particularly the remarkable mechanisms that lead to the generation of specific and cross-reactive antibodies. Along the way, I highlight parallels between different immunological processes, such as TCR activation in the immunological synapse and Fc-receptor activation in the phagocytosis cleft. Understanding these fundamental mechanisms can offer insights applicable to other areas of immunology. Chapter 2 focuses on antibodies, emphasizing their dynamic nature and the complex interactions that mediate their function. This knowledge forms the foundation for the development of clinical therapeutics, which leads naturally into Chapters 3 and 4, covering the discovery and engineering of monoclonal antibodies. To determine which antibody modifications are beneficial and which antigens should be targeted, it is crucial to study host-pathogen interactions through both clinical/epidemiological and preclinical research. This rationale is reflected in Chapters 5 and 6, which explore *Streptococcus pyogenes* and SARS-CoV-2, respectively. Finally, the thesis culminates in Chapter 7, where these elements come together in the present investigation. Now that this journey is coming to an end, I find myself with even more questions about how antibodies mediate their effects. There is still so much to learn about harnessing the potential of monoclonal antibodies to treat infectious diseases. But as I look ahead to an uncertain future, I find excitement in the unknown something that reminds me of a quote from one of my favorite books (arguably one of the greatest literary works of all time):

“It's a dangerous business, Frodo, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to.”

- J.R.R. Tolkien

Author disclaimers: I the author take full-responsibility of the content in this thesis. The text, with the content solely produced by the author, has been analyzed by AI-tools to easier spot grammar issues and spelling errors, and when applicable edited for better using suggestions provided by said AI-tools. The cover-photo is generated by using a model template that was created initially to generate MD simulations from paper II (see figure 2G) and edited using adobe illustrator to then generate additional color with “cool” effect by the AI-tool Adobe Firefly for better graphical design. Some of the antibodies generated in this thesis by the author and others are potentially of interest for future development as therapeutics. Several patents are filed and a spin-off antibody company has been formed to potentially commercialize these antibodies when applicable, where the author is a co-founder and will take a potential active role.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Den mänskliga kroppen är sammansatt av flera organsystem som arbetar tillsammans för att möjliggöra liv. Immunförsvaret är ett av dessa system och har som huvuduppgift att skydda oss mot infektioner som orsakas av bland annat virus och bakterier. Immunförsvaret består bland annat av celler som cirkulerar i kroppen och har förmågan att fagocytera, eller "äta upp", virus och bakterier (sjukdomsframkallande mikroorganismer som kallas patogener). Andra specialiserade celler kan döda de mänskliga celler som blivit infekterade av dessa patogener.

När en infektion inträffar tränas immunförsvaret, och det bildas celler som "minns" patogenerna. Detta gör att kroppen kan reagera snabbt och effektivt om samma patogen skulle infektera oss igen. En särskilt viktig del av detta försvar är B-cellerna, som producerar Y-formade proteiner, kallade antikroppar. Dessa antikroppar binder till patogeners yttre strukturer, vilket gör att de markeras för eliminering av immunsystemet. När antikropparna binder till en patogen, signalerar deras stam till immunceller att patogenen ska tas om hand genom fagocytos. Denna process, där antikropparna gör patogenerna "aptitretande" för fagocyter, kallas opsonisering, ett grekiskt ord som betyder "att göra aptitretande."

Antikroppar fungerar alltså som väldesignade målsökande proteiner som vägleder immunceller till att eliminera inkräktarna. De är centrala för den så kallade förvärvade immuniteten, vilket innebär att kroppen efter en första infektion lär sig att känna igen och hantera patogener. Immunförsvaret kan tränas genom exponering för ofarliga versioner av patogener, vilket är principen bakom vaccinering. Vacciner består ofta av döda eller försvagade virus, eller till och med bara delar av viruset, för att stimulera immunförsvaret att skapa ett skydd.

Genom att studera hur immunförsvaret designar antikroppar mot virus och bakterier kan vi utveckla läkemedel i form av monoklonala antikroppar som kan användas för behandling av svåra infektioner. Med dagens teknologi kan vi skapa och testa antikroppar i laboratorier för att utvärdera om de är effektiva, till exempel genom att få immunceller att fagocytera bakterier eller virus. En stor fråga inom forskningen är vad som gör en antikropp effektiv. Är det hur starkt den binder till patogenen, var på patogenen den binder, eller är det hur antikroppen interagerar med immunceller som spelar störst roll? Dessa frågor är fortfarande komplexa och kräver omfattande grundforskning för att förstå hur vi kan utveckla effektiva läkemedel.

Under COVID-19-pandemin fick antikroppsbehandlingar stor uppmärksamhet. Ett okänt virus från coronavirusfamiljen spred sig världen över och orsakat miljontals

dödsfall. Vårt immunförsvar producerar antikroppar mot virusets spike-protein, som viruset använder för att ta sig in i våra celler och föröka sig. Antikroppar som binder till spike-proteinet och blockerar virusets inträde kallas neutraliserande antikroppar. Dock utgör dessa en liten del av de antikroppar som riktar sig mot spike-proteinet, och det är oklart om antikroppar som binder till viruspartiklar och underlättar deras upptag och eliminering genom fagocytos också ger skydd. De flesta kommersiella antikroppsbehandlingar mot COVID-19 har fokuserat på att neutralisera viruset, utan att beakta den potentiella skyddande effekten av opsonisering. Ett av målen i denna avhandling har varit att undersöka om opsoniserande antikroppar kan skydda mot svår sjukdom och om vi kan skapa ännu mer effektiva antikroppar som aktiverar immunceller för att underlätta fagocytos.

I denna avhandling visar vi att icke-neutraliserande antikroppar som kan aktivera immunceller att fagocytera viruspartiklar (som är märkta med spike-proteinet) skyddar möss mot en dödlig dos av det ursprungliga Wuhan-viruset. Denna skyddande effekt är jämförbar med neutraliserande antikroppars effekt i samma djurmodell. Dessa resultat stöds av andra forskargrupper som har visat att dessa icke-neutraliserande men opsoniserande antikroppar ger skydd både hos människor och i djurmodeller. Vi undersökte även hur antikropparnas struktur påverkar deras effektivitet och fann att en variant av antikropparna, där en del av deras struktur byttes ut, ledde till starkare aktivering av immunceller och en förbättrad bindning till spike-proteinet.

Vidare visade vi att dessa antikroppar behöll sin skyddande funktion även mot de muterade varianterna Omicron och JN.1, som har dominerat i spridningen av viruset och uppvisar lägre dödlighet än det ursprungliga Wuhan-viruset. Därmed visar denna avhandling att icke-neutraliserande antikroppar kan ge starkt skydd mot virus, även mot muterade varianter, och att deras funktion kan förbättras genom att justera deras struktur.

En annan infektion som blivit mer uppmärksammad efter pandemin är Grupp A streptokockinfektioner, som normalt orsakar halsfluss men som kan ge allvarliga sjukdomar som blodförgiftning och köttätarsjuka. Denna bakterie använder ett protein, M-proteinet, för att undvika immunförsvaret. Vår forskargrupp har utvecklat en antikropp, Ab25, mot detta protein som skyddar möss mot allvarlig sjukdom. Vi undersökte de olika IgG-klasserna av Ab25 och fann att IgG3-versionen av Ab25, trots att den band svagare till M-proteinet, var mycket mer effektiv på att aktivera immunceller att fagocytera bakterier.

Genom avancerad datasimulering kunde vi visa att IgG3-antikroppar rörde sig mer i det tredimensionella rummet relativt till bakterien, vilket underlättade för

immunceller att hitta bakterien. Vi skapade även en hybridantikropp som kombinerade delar av både IgG1 och IgG3, och denna hybrid visade sig ha en överlägsen förmåga att aktivera immunceller och att skydda mot spridning av bakterier till andra organ i en djurmodell som simulerade allvarlig streptokockinfektion.

Den starka effekten hos denna nya klass sågs även mot andra grupp A streptokock-stammar som infekterade patienter. Slutligen visade vi att denna nya antikroppsstruktur också förbättrade opsoniseringseffekten hos icke-neutraliserande antikroppar mot COVID-19. De förbättrade antikropparna visade ökad effektivitet även mot andra virusstammar inom coronavirusfamiljen. Denna generella effekt verkar bero på att stammen binder bättre till immuncellernas proteiner som känner igen antikroppar, vilket underlättar deras aktivering för att eliminera patogener.

Sammanfattningsvis visar denna avhandling att vi kan utveckla modifierade antikroppar för att bekämpa både streptokocker och coronavirus genom att öka immuncellernas förmåga att eliminera patogener. Den nya hybridklassen av antikroppar utgör en lovande strategi för framtida antikroppsbehandlingar och har potential för klinisk användning för att behandla infektioner från både bakterier och virus.

ABBREVIATIONS

mAb -	Monoclonal antibody
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcR	Fc receptor
CD	Cluster of differentiation
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity/antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ICAM-1	Intercellular Adhesion Molecule 1
VCAM-1	Vascular Cell Adhesion Molecule 1
LFA-1	Lymphocyte function-associated antigen 1
CR3	Complement receptor 3
CR4	Complement receptor 4
AMP	Antimicrobial peptides
PRR	Pattern recognition receptor
IgG	Immunoglobulin G
MHC	Major Histocompatibility Complex
NK	Natural Killer
STSS	Streptococcal toxic shock syndrome
SAg	Superantigens
TCR	T-cell receptor
BCR	B-cell receptor

CDR	Complementarity determining region
SHM	Somatic hypermutation
CSR	Class-switch recombination
GC	Germinal center
SMAC	Supramolecular Activation Complex
PAMP-	Pathogen-associated molecular patterns
MOP	Multiplicity of prey
IC_{50}	Concentration to reach 50% inhibition
KD	Dissociation constant
SPR	Surface plasmon resonance
MD	Molecular dynamics
SLO	Streptolysin O
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
COVID-19	Coronavirus disease 2019
NSTI	Necrotizing soft-tissue infection

LIST OF PAPERS

Publications and manuscript part of this thesis

Paper I
Izadi, A., Hailu, A., Godzwon, M., Wrighton, S., Olofsson, B., Schmidt, T., Söderlund Strand, A., Elder, E., Appelberg, S., Valsjö, M., Larsson, O., Wendel-Hansen, V., Ohlin, M., Bahnan, W., & Nordenfelt, P. (2023). Subclass-switched anti-Spike IgG3 oligoclonal cocktails strongly enhance Fc-mediated opsonization. *Proceedings of the National Academy of Sciences*, *120*(15).

Paper II
Izadi, A., Karami, Y., Bratanis, E., Wrighton, S., Khakzad, H., Nyblom, M., Olofsson, B., Happonen, L., Tang, D., Sundwall, M., Godzwon, M., Chao, Y., Gomez Toledo, A., Schmidt, T., Ohlin, M., Nilges, M., Malmström, J., Bahnan, W., Shannon, O., Malmström L., & Nordenfelt, P. (2024). The hinge-engineered IgG1-IgG3 hybrid subclass IgGh47 potently enhances Fc-mediated function of anti-streptococcal and SARS-CoV-2 antibodies. *Nature Communications*, *15*, 1-22.

Paper III
Izadi, A., Godzwon, M., Söderlund Strand, A., Schmidt, T., Kumlien Georén, S., Drosten, C., Ohlin, M., & Nordenfelt, P. (2024). Protective Non-neutralizing anti-N-terminal Domain mAb Maintains Fc-mediated Function against SARS-COV-2 Variants up to BA.2.86-JN.1 with Superfluous in vivo Protection against JN.1 Due to Attenuated Virulence. *Journal of immunology*, *213*(5), 678-689.

Paper IV
Izadi, A. Godzwon, Yanartas, D. Hultqvist, A. Mats Ohlin, Pontus Nordenfelt. Hinge-engineered IgGh47 monoclonal antibodies exhibit ultrapotent Fc-mediated phagocytosis against SARS-CoV-2 and Betacoronaviruses through an unprecedented high-affinity binding to CD32 and CD16. ***Manuscript*** *(preliminary author list)*

Publications *not* part of this thesis

Review

Izadi, A., & Nordenfelt, P. (2024). Protective non-neutralizing SARS-CoV-2 monoclonal antibodies. *Trends in Immunology*, *45*(8), 609-624.

Letter to editor

Izadi, A., Bahnan, W., Ohlin, M., & Nordenfelt, P. (2023). Reply to Pandey: IgG3 allotypes, modulation of antigen binding by constant domain changes, and therapeutic applications of Fc engineering. *Proceedings of the National Academy of Sciences*, 120(22), 1

Published research articles

Gomez Toledo, A.; Chowdhury, S.; Hjortswang, E.; Sorrentino, J. T.; Lewis, N. E.; Bläckberg, A.; Ekström, S.; Kjellström, S; **Izadi, A**.; Olofsson, B.; Nordenfelt, P.; Malmström, L.; Rasmussen, M.; Malmström, J. (2024). Dissecting the Properties of Circulating IgG against Streptococcal pathogens through a combined systems antigenomics-serology workflow. *Nature communications* , 16, 1-16

A past reminder of future potential

"We can only support each other mutually, just as phagocytes and antitoxins do, since it can certainly be assumed that phagocytes receive major help from antitoxic activity, and that phagocytes, by capturing and destroying bacteria, provide major help for the host organism regarding its antitoxic defense"

- Elie Metchnikoff in a letter to Emil Behring (1906)[1]

I choose to highlight the quote above because it is one of the crucial exchanges between the founders of Immunology as a field- Metchnikoff who discovered phagocytosis and Behring who discovered the presence of antitoxin in serum. What they discovered was that there exist unique and specialized components in the human body which eliminate invasive pathogens through distinct mechanisms. The human body and the intricate and sophisticated systems existing have been developed over millions of years of evolution. In the case of the immune system, it can generate an organized and specific response against invading intruders through complex biological mechanisms which are highly regulated. It is only in the last 150 years that we humans have discovered and keep discovering these mechanisms but also how to harness them in the form of medicine to combat these invading pathogens. The purpose of this thesis is to build on the foundations set by giants such as Emil Behring and Elie Metchnikoff and increase our understanding of how we can harness the immune system's most sophisticated arsenal against pathogens - *antibodies*. This is highlighted by a 130 year old quote by Behring when he discovered that antitoxins (antibodies) in serum can be used as treatment against Diphtheria toxin:

"The possibility of cures for even highly acute diseases can thus no longer be ignored"- Emil Behring (1890)[1]

CHAPTER I
THE TWIGS AND BRANCHES OF THE IMMUNE SYSTEM

Before we delve deep into the fascinating world of antibodies and their use as monoclonal antibodies in the medical field, we will have a glance at the big tree of which they are an important branch. We will begin by starting with the major branch known as the *innate immune system***,** responsible for 1) preventing infection and maintaining homeostasis 2) eliminate the invaders and 3) calling in the *adaptive immune system* consisting of the fascinating T-and B-cells. We will briefly discuss these two systems from the point of an infection (bacterial and viral infection, sorry parasites and fungus) as to set the stage for the work presented in this thesis. The chapter is structured to be more superficial in the beginning and subsequently more detailed as we progress.

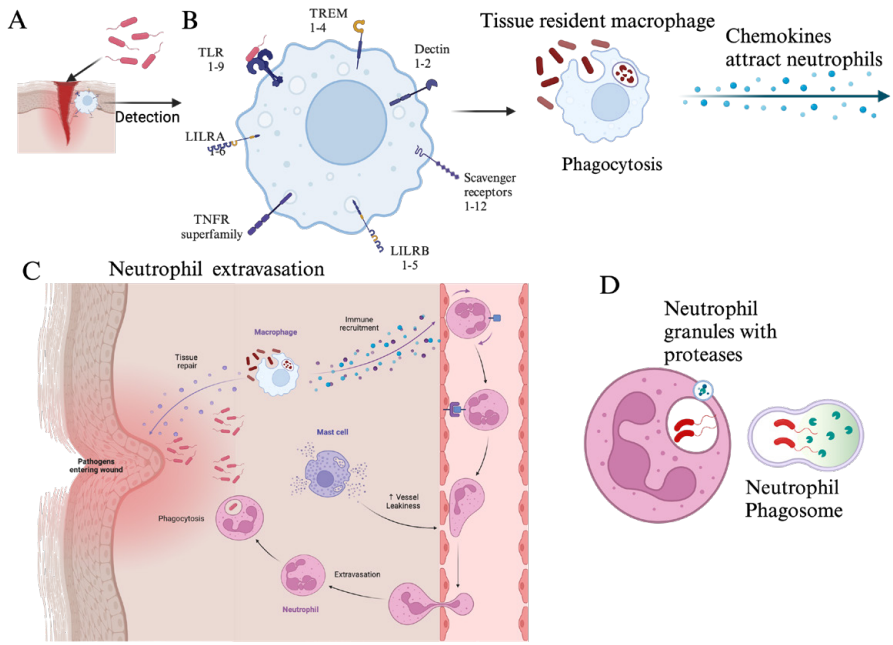

Figure 1. Overview of the innate immune response and key players. A Bacterial invasion through a skin breach is detected by resident macrophages. **B** Macrophage receptors ingest bacteria via phagocytosis, releasing chemokines. **C** Neutrophil extravasation to the inflammation site, mediated by chemokines. **D** Neutrophil phagosome formation with antimicrobial substances from fused granules. Created with BioRender (**C** by Danielle Penk and Louis Ngai).

THE INNATE IMMUNE SYSTEM

YOU SHALL NOT PASS: THE IMPORTANCE OF A STRONG PHYSICAL BARRIER

By far the largest part of the innate immune system is the skin organ of humans, which constitutes both a physical and chemical barrier to invading pathogens[2]. The skin is a barrier, which in the outermost epidermal layer, is made up of specialized epithelial cells, called keratinocytes, which are joined together through protein complexes called tight junctions, which is one reason why pathogens cannot easily slip past the epithelial cells. Similar defense mechanisms are found in other epithelial barriers, including those in the pharynx, nose, trachea, lungs, urogenital tract, and gut. The physical barrier is further strengthened by having a slippery watery substance, similar to egg white, called mucus which covers the epithelial cells[3]. The mucus is made up mainly of water and proteins called mucins, which give it viscosity and are produced by neighboring mucosal glands. The pathogens invading the trachea for instance would get stuck in the mucus and not be able to adhere to the epithelial barrier and disrupt the integrity of the barrier. Furthermore, the mucus and the trapped pathogens are removed by physical movement of cilia, in the trachea in this example.

The importance of these physical barriers or the physical removal of pathogens by mucus is highlighted in two distinct medical diseases called cystic fibrosis (CF)[4] and atopic dermatitis (AD) [5]. In CF, patients have a mutation in a protein responsible for transportation of water and ions to the outside of the epithelial cells leading to thickening of the mucus. This leads to impaired clearance of mucus by cilia organelles and build up of uncleared mucus which leads to chronic infection with fungal and bacterial pathogens (of which pseudomonas aeruginosa is one). In AD patients, amongst other things, have disrupted skin barrier which enables a common colonizer of our skin flora, Staphylococcus aureus, to cause skin infection in otherwise healthy individuals[5] .

WHEN PHYSICAL OBSTACLES ARE NOT ENOUGH FIGHT TO INTRUDERS WITH A HOSTILE ENVIRONMENT- THE CHEMICAL BARRIER

One aspect of the skin, lung and guts defenses against invading pathogens is the chemical component of the barriers. The chemical component is constituted by a broad range of diverse molecules, ranging from small proteins such as antimicrobial peptides such as beta-defensin[2] to acids in the gut such as hydrochloric acids[6] . Beta-defensins, produced by keratinocytes (among other cells), disrupt the integrity of bacterial membranes, leading to lysis/death. In addition beta-defensins can interact with the adaptive immune system by recruiting more specialized cells through

chemotaxis for instance[2] . Helicobacter pylori is one bacterial pathogen which can colonize the human gut and escape elimination by acid; it does this by a surgical-pinpoint strike by neutralizing the acid by ammonia production thereby escaping the defense of this chemical barrier[6] . The host has developed these complex systems, and the pathogens have co-evolved countermeasures to escape them and thereby breach the first barriers. But luckily for us, this was just the first obstacle for the pathogens and the human body has many surveillance systems[7] to find out when a breach has occurred which we will discuss shortly below.

SPOTTING INTRUDERS AND SOUNDING THE ALARM

Despite the everyday success of the chemical and physical barriers in keeping out invading pathogens and maintaining balance with the microbial flora, breaches leading to infections do unfortunately occur (**Figure 1A**). Therefore, a reliable detection system is crucial to signal when the body is under attack[7]. But to have a suitable detection system, the system needs to reliably distinguish between what is foreign and what belongs to the "self". Bacteria, viruses, parasites and fungal pathogens have unique molecules and structures which are not produced by human cells. These molecules are called pathogen-associated molecular patterns (PAMP) and they differ from pathogen to pathogen, but what is common for all of them is that they are distinct from molecules produced by the human host and that they can be recognized by our immune system's different detectors[7–9] . Bacterial examples of these PAMP's are the peptidoglycan wall of gram positive bacteria or the lipopolysaccharide acid (LPS) of gram negative bacteria. These unique signatures are detected by specific tools developed by the human body.

The body has developed intricate detection tools which can recognize these various foreign PAMP's by so-called Pattern recognition receptors (PRR's) (**Figure 1B**)[7] . PRR's are localized on cell-membranes (on epithelial cells for instance) belonging to the toll-like receptor family (TLR's). Intracellularly they can exist in the cytosol in the form of retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)- like receptors (NLR's). The different receptors recognize different PAMP's on different pathogens, thus complementing each other while some are more broad such as Toll-like receptor 2 can recognize both viral, bacterial, fungal and protozoan PAMPs[7–9] . When PAMPs bind to PRRs, an intracellular alarm signal is activated. This signal triggers a cascade of intracellular molecules, leading to the synthesis of inflammatory proteins. One example of this is the detection of the envelope protein (E protein) of SARS-COV-2 virions by TLR2 which leads to a downstream production and secretion of interferon-γ cytokine [10]. Interferon-γ has antiviral effects and enhances

antigen presentation by increasing the expression of Major Histocompatibility Complex (MHC) class II proteins[11] . MHC class 2 proteins have a crucial role in displaying proteins of pathogens to specialized immune cells of the adaptive branch of the immune system. The cells that display the pathogen proteins as peptides on MHC class 2 molecules for the adaptive immune cells are called antigen-presenting cells and they will be discussed more later[11]. One important effector function which is elicited by PRR stimulation by PAMP's is secretion and production of cytokines such as tumor necrosis factor α (TNF-α) and chemokines such as interleukin-8 (IL-8/CXCL8) which will attract the attention of the innate immune systems effector cells such as neutrophils and macrophages. These are the important foot-soldiers of the innate immune systems and will be further discussed.

TISSUE RESIDENT MACROPHAGES - SURVEILLING SENTINELS

Once a pathogen has breached the chemical and physical barrier, it will be recognized by the immune systems sentinels which are spread throughout the body[12]. These are known as tissue resident macrophages (**Figure 1B**). In the lungs, in alveoli, they are called alveolar macrophages and are important for phagocytosis of pathogens in that niche in addition to specialized functions unique for the alveoli such as removing surfactant produced by its neighboring cells[12] . Regardless of whether they are in the lungs or skin, these tissue resident macrophages recognize invading pathogens PAMP's through their PRRs such as toll-like receptors (TLR). Upon recognizing their ligands, such as TLR2 recognizing the envelope protein of SARS-COV-2 virus (for instance) [10], they engulf (phagocytose) the pathogen to clear the infection as damage control. Not only that, as discussed above, the activation of PRRs also lead to intracellular signaling which lead to production of cytokines (interferon-γ, TNF-α) and chemokines (IL-8) which attract other immune cells to help combat the pathogens and activate the cells to more efficiently eliminate the pathogens who have breached through the system. Macrophages in the tissue are also important for healing the wound and sealing the breach once the infection has been cleared. These cells develop in the bone marrow from immature monocytes, differentiating into macrophages with a pro-healing phenotype[12]. The immune cells that are recruited by tissue resident macrophages are initially the patrolling foot-soldiers called neutrophils which are the most abundant white blood cells in blood[13]. Neutrophil mobilization to the inflamed/infected site by chemo-and cytokines are followed by a later-stage mobilization of immature monocytes from the bone-marrow which are differentiated into a pro-inflammatory macrophage (can also become an anti-inflammatory macrophage) which helps the neutrophils in eliminating the pathogen.

The importance of having functioning neutrophils is evident in immune-deficient patients lacking this cell-type, where they overwhelmingly acquire infections. Neutrophils are the most abundant white blood cells in the human body and are generated in the bone marrow. Neutrophils are classified as granulocytes (along with basophils and eosinophils) due to them containing preformed granules in their cytoplasm[13] .

Once released into the bloodstream, neutrophils can migrate to the site of injury through a process called extravasation. At the site of damage/breach/infection, endothelial cells (which make up the blood vessels) will express certain ligands (selectins) which neutrophils will bind to through its corresponding receptor (mucin-like molecules), this first step is known as tethering and leukocyte rolling[14] . The endothelial cells will express these ligands as a response to chemokines (TNF-α, IL-1 etc) released by cells such as tissue-resident macrophages (**Figure 1C**). Subsequently, a second activation, triggered by chemokine molecules (produced by the endothelial cells) binding to chemokine receptors present on neutrophils, will activate a family of proteins called integrins (such as LFA-1, MAC-1) on neutrophils. Then a second activation by the chemokine molecules (produced by the endothelial cells) to chemokine receptors present on neutrophils will activate a family of proteins called integrins (such as LFA-1, MAC-1) on neutrophils. Integrins which are present on the cell-membrane in a default low-affinity state (affinity state relative to its receptor) will change confirmation to a high affinity-state enabling strong binding to corresponding receptors (ICAM-1, also known as CD54) on endothelial cells, resulting in adherence[14] . Once adhered to the endothelial wall, the neutrophil will squeeze through the cellular-junctions through a process called diapedesis and will be guided to the presence of the pathogens by chemokines released by the sentinel immune cells in the local tissue[13] .

Neutrophils have three key functional features which distinguishes them from macrophages and other effector cells. First, although not unique in this ability, neutrophils are highly efficient phagocytes, capable of engulfing pathogens effectively[13] . The process of phagocytosis is initiated by the neutrophil once it recognizes the foreign pathogen. There are several unique receptors mediating this interaction. A notable example is the pattern recognition receptors (PRRs) which recognize specific motifs that are commonly expressed on pathogen proteins for instance. Other receptor types involved are complement receptors or Fc-receptors. These receptors recognize complement proteins and antibodies which are deposited on the pathogen, marking them for destruction and subsequent engulfment by neutrophils (more on this later). Once a pathogen is recognized by the neutrophils

receptor/receptors, the process of phagocytosis will take place where parts of the cellular membrane will engulf the pathogen, forming the phagocytic cup, and then being pinched off and form a membrane vesicle called phagosome[15] . The phagosome containing the pathogen will fuse with neutrophil granules which are loaded with proteins made specific to kill the pathogen (such as hydrolytic enzymes). Although other phagocytes such as macrophages engulf pathogens in a similar manner, there are important features in the neutrophil which differ once the phagosome enters the cytosol. Unlike macrophages, neutrophils have pre-made granules (or vesicles) containing different proteins which have specialized antimicrobial activity[15–18] . The content of the granules are produced already when the neutrophil is differentiating in the bone marrow, so when it encounters the pathogen in the breached injury site (in the skin for instance) it has come prepared for the battle. There are four types of vesicles/granules: Azurophilic, specific, gelatinase and secretory granules. The azurophilic granules and specific granules both contain antimicrobial proteins while gelatinase vesicles have proteins which can remodel the extracellular matrix[15–17] . Finally the secretory vesicles contain mostly plasma proteins and membrane receptors which is important for phagocytosis for instance. Once a pathogen is engulfed by a neutrophil and isolated in a phagosome, the azurophilic and specific vesicles fuse with the phagosome to achieve antimicrobial killing by releasing their antimicrobial proteins (such as myeloperoxidase, elastase, cathepsin G and more). Due to the existence of these granules in large quantities and the faster formation of the mature neutrophil phagosome, neutrophils are viewed to have greater capacity of eliminating pathogens through phagocytosis than macrophages which lack these preloaded vesicles (**Figure 1D**)[13] .

The second feature, relating to phagosome killing, is that neutrophils have a high degree of expression of a protein complex called Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, known as NOX2 in humans)[19] . Through the activity of this protein complex, radical oxygen species (ROS) can be produced inside the lumen of phagosomes. ROS are molecules containing an oxygen-component which interacts and disrupts pathogens proteins critical for survival which leads to the death of the invader. Neutrophils are not unique in their ability to produce ROS for antimicrobial activity, but they are known to be more potent producers of ROS than macrophages and monocytes. It is postulated that one reason for the higher pH in a mature phagosome of a neutrophil as compared to macrophages is due to much more potent ROS production by NADPH oxidase in neutrophils. Interestingly, proteins in azurophilic granules such as MPO can potentiate the effect of ROS produced by the NADPH oxidase (by catalyzing the

formation of hypochlorous acid, a powerful oxidant), thus having synergy in eliminating the pathogen[20] .

While phagocytosis by neutrophils, along with the fusion of intracellular granules with the phagosome and ROS production by NADPH oxidase, is crucial for intracellular pathogen killing, neutrophils also possess a unique extracellular defense mechanism. Neutrophils can decondense their tightly packed DNA, combine it with granular proteins, and release this network extracellularly to trap and kill pathogens like bacteria and fungi[21] . These structures, known as Neutrophil Extracellular Traps (NETs), are a key component of the neutrophil's arsenal against foreign invaders. However, maintaining a balance between sufficient pathogen killing and minimizing collateral damage is critical; excessive release of NETs or ROS can harm host tissues and trigger excessive inflammation with detrimental clinical consequences[22,23] . For example, in the case of SARS-CoV-2, excessive NET release by neutrophils in the lungs has been associated with severe disease outcome. Nevertheless, neutrophils play a critical role in locating infection sites-guided by signals from dendritic cells, tissue-resident macrophages, and other cells and clearing infections primarily through phagocytosis and NET release. If neutrophils are unable to clear an infection on their own, they can stall invaders long enough for reinforcements such as monocytes from the bone marrow to arrive.

MONOCYTES- THE SWISS ARMY KNIFE OF THE INNATE IMMUNE SYSTEM

While neutrophils are known to be the first responders at the site of inflammation and also the most abundant white blood cell in the bloodstream, they do not last long due to their short-lived nature[13] . While the neutrophils which have entered the inflammation site are occupied with phagocytosing the pathogen, monocytes are being produced at the bone marrow. Once released into the bloodstream, monocytes will be attracted to the inflamed site by the presence of chemokines and extravasate like neutrophils through integrin-based adhesion through the endothelium[24] . Unlike neutrophils and tissue-resident macrophages, monocytes are comparatively immature and not as efficient in mediating phagocytosis compared to them. However, the plasticity of monocytes should not be underestimated, by being uncommitted, monocytes can differentiate into the type of cell needed for the task at hand. During homeostasis, monocytes can enter the liver, spleen, skin, testis and other organs to differentiate into macrophages with similar function as the local tissue-resident macrophages (which were engrafted during embryonic development and evidence shows that they have capacity for self-renewal)[25] .

During inflammation and infection, monocytes can differentiate into what has been historically categorized as M1 or M2 macrophages, a process which is called polarization[26] . This classification is based on the phenotype of having either a pro or anti-inflammatory function and the polarization to either of them is influenced by environmental cues such as cytokines produced at the site of inflammation. Monocytes differentiate into M1 macrophages through cytokines such as Interferon-γ produced by the sentinel cells such as tissue-resident macrophages (**Figure 2A**). Interestingly, pathogen associated structures such as lipopolysaccharide on bacteria can also differentiate monocytes into M1 macrophages through recognition by the Pathogen pattern receptor TLR4[27] . The "classical" view is that during early stages of an infection, monocytes are differentiated into M1 phenotype to be able to recruit more immune cells through production and release of proinflammatory cytokines (such as tumor necrosis factor, interferon-α), phagocytose pathogens, produce ROS and nitric oxide (which is important for phagosomal killing) and coordinate with the adaptive immune cells (T-cells more specifically, more on this later)[24] . However, once the fight is over it is crucial to heal the damaged tissue site to prevent another infection and regain function of the tissue. It is in this context where cytokines such as IL-4 will cause the monocytes to polarize to M2 phenotype, which will be important for wound-healing, through production of collagen for instance (an important part of the extracellular matrix)[24–26] . One important function the tissue-resident macrophages have and the M1 macrophages, is that they can activate natural-killer cells (NK-cells) through interferon-α cytokine release[28] . The importance of NK-cells will be discussed below.

NK-cells: The main effector cell for Antibody Dependent Cellular Cytotoxicity

While neutrophils and macrophages are potent phagocytes where they clear extracellular pathogens, natural killer (NK) cells play a crucial role in eliminating infected cells during the early immune response[29,30]. Although it is widely considered to be important for antiviral defense, NK-cells also contribute significantly to the immune response against intracellular bacteria and tumors. Upon infection by a viral pathogen, host cells undergo changes that trigger NK cell-mediated elimination. These changes include the release of cytokines (such as IL-2 and interferon-α), which activate NK cells, leading to proliferation, increased production of cytotoxic granules (vesicles containing proteins that induce cell death via apoptosis), and enhanced NK cell survival (**Figure 2B)**[28,30] . In addition to cytokine production, infected host cells often downregulate major histocompatibility complex class I (MHC-I) molecules, triggering NK cell-mediated killing through degranulation[31] . MHC-I molecules present self-antigens, signaling

"self" to NK cells; downregulation of MHC-I prevents this recognition, allowing NK cells to identify and eliminate infected cells. Another key effector function mediated by NK-cells is antibody-dependent cellular cytotoxicity (ADCC) through CD16a/Fcγ3A binding of Immunoglobulin constant domain (see **Chapter 2** for details)[32] . Macrophages activate NK cells through the release of interferon-α.[33] In turn, activated NK cells are major producers of interferon-γ, a cytokine that enhances macrophage polarization into the pro-inflammatory M1 phenotype, upregulates MHC-II expression for antigen presentation, and recruits additional immune cells to the site of infection[30]. Thus, NK cells eliminate infected cells, promote a stronger inflammatory phenotype in neighboring cells, and help coordinate the innate immune system to activate antigen-presenting cells of the adaptive immune system. Recent research is showing that a subset of NK-cells can acquire memory-like functionality (see review by Cewanka and Lanier)[30]. The most efficient antigen presenting cell, the dendritic cell, will be further explained below.

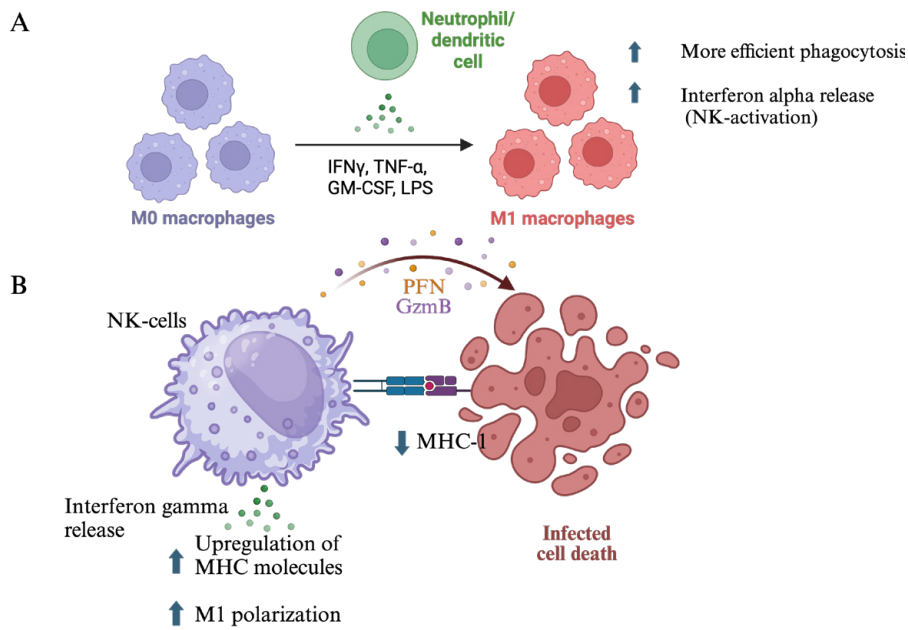

Figure 2. Overview of Macrophage Polarisation and NK-cell Killing of Infected Cell. **A** Polarization of macrophages to the M1 phenotype induced by cytokines and other environmental cues. **B** Illustration of NK cell-mediated killing of an infected cell. Created with BioRender.

DC-cells (Dendritic cells)

While macrophages and neutrophils work to eliminate foreign pathogens, these responses may be insufficient for complete clearance and long-term protection. The adaptive immune system, primarily mediated by T and B cells of the lymphocyte lineage, generates specialized immune responses targeting unique proteins and virulence factors of pathogens (molecules critical for host infection and pathogen survival). Furthermore, the adaptive immune system establishes immunological memory, enabling faster and more efficient pathogen clearance upon subsequent encounters - a phenomenon known as immunity. To be able to generate an adaptive immune response against a pathogen, the T and B cells need to recognize the foreign proteins and other structures of the pathogen. Dendritic cells (DCs) play a crucial role in presenting these antigens to naive T and B cells[34] .

Upon encountering foreign bacteria in the skin (where they reside as tissue-resident DCs), dendritic cells recognize bacterial proteins and structures through pattern recognition receptors (PRRs), leading to phagocytosis and subsequent processing in the phagolysosome[34] . Within the phagolysosome, bacteria are digested into smaller components[35]. Concurrently, MHC-II molecules are produced in vesicles that fuse with the phagolysosome, enabling the capture of bacterial peptides (smaller protein segments). These peptide-MHC-II complexes are then transported to the dendritic cell's surface. Once captured, the MHC-2 molecules and the pathogens peptide will be taken to the cell-membrane of the dendritic cell[34]. During this process, the dendritic cell migrates to lymph nodes to interact with T cells. The initial encounter with bacteria triggers these critical steps, where PRR engagement initiates not only phagocytosis but also intracellular signaling pathways (such as the NF-κB pathway). These activated signaling pathways increase the production of MHC-II proteins, enhance antigen processing in phagolysosomes (via proteases), produce T cell-activating co-stimulatory factors, and induce chemokine production, which guides dendritic cell migration to lymph nodes via lymphatic vessels (detailed further below).

How are Viruses or intracellular pathogens presented?

The preceding example illustrates how extracellular bacteria are presented to T cells via MHC-II molecules[36] . However, how are viruses which infect host cells, including dendritic cells presented to T cells? As previously noted, MHC-I molecules present self-antigens. If a dendritic cell is infected by a viral pathogen and its protein machinery is co-opted to produce viral proteins, these viral proteins are also loaded onto MHC-I molecules and transported to the cell surface[36] . This process is enhanced by cytokines such as interferon-γ produced by neighboring cells

(such as NK-cells) or upon recognition of viral motifs by intracellular PPRs. Nevertheless, once the infected cell displays the foreign peptide on MHC-1, the cytotoxic T-cells (CD8+ cells) can directly be activated without the engagement of helper T-cells (CD4+). Interestingly, dendritic cells can also phagocytose pathogens without its intracellular machinery being hijacked. By engulfing the pathogens through phagocytosis and degradation of proteins into peptides in the phagolysosome, the content can be loaded on MHC-1 molecules and be brought to the cell-membrane through this pathway[34] . This alternative pathway is known as *cross-presentation* and can directly activate CD8+ T-cells without CD4+ mediation. In summary, dendritic cells are essential for initiating adaptive immune responses by presenting protein-derived peptides on MHC-I and MHC-II molecules following phagocytosis, thereby engaging CD4+ and CD8+ T cells (**Figure 3A**). The interaction between T cells and dendritic cells, known as the immunological synapse, will be examined further in the subsequent section on the adaptive immune system.

THE ADAPTIVE IMMUNE SYSTEM: LEARNING BY DOING

The innate immune system provides a rapid initial response to foreign pathogens, whereas the adaptive immune system orchestrates a targeted and coordinated attack involving both cellular and humoral components to eliminate infections. B-cells produce antibodies, Y-shaped proteins secreted into mucosal areas and the bloodstream, that bind to specific structures (antigens) on pathogens and mediate much of humoral immunity[37]. T-cells are the primary mediators of cell-mediated immunity, recognizing antigens via their specific T-cell receptors (TCRs)[38] . The specificity of antibodies and TCRs is due to a vast repertoire of genes that encode the antigen-binding regions of these receptors. This receptor diversity is further amplified by combinatorial rearrangement of the genes that encode the variable regions of TCRs and B-cell receptors (BCRs)[39]. To fully appreciate the adaptability of B- and T-cells, we will now examine how these receptors are generated to recognize a broad range of pathogens and their antigens.

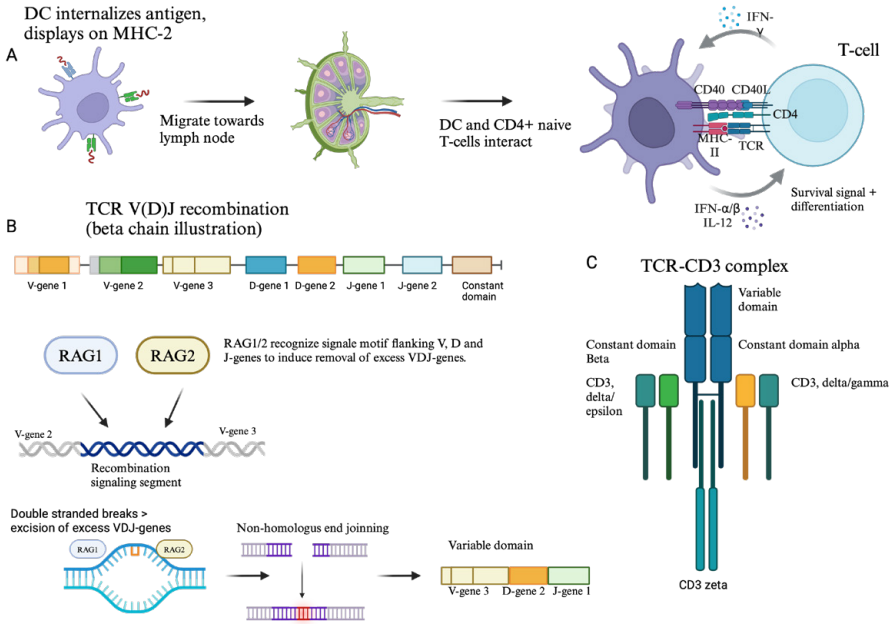

Figure 3. T Cell Activation by Dendritic Cells and Structure of TCRs. A Overview of dendritic cell-mediated T cell activation. **B** V(D)J recombination generating the variable domain of the TCR. **C** Simplified structural overview of the TCR complex, illustrating co-receptors. Created with BioRender.

T-CELLS

T-cells originate from the bone marrow, and their progenitors migrate to the thymus for maturation[40,41]. These progenitors initially lack CD4 and CD8 co-receptors but acquire both during maturation (double positive progenitors) before differentiating into either helper (CD4+) or cytotoxic (CD8+) T-cells respectively. During T-cell maturation, the cell will acquire both receptors (double positive precursors) and later dedicate itself to become either a CD4+ T-cell or CD8+ T-cell. These co-receptors pair with the TCR, generated in the thymus where the T-cell receptor passes through a process called *T-cell receptor rearrangement*[42] .

T-CELL RECEPTOR STRUCTURE

The T cell receptor (TCR) consists of two heterodimeric chains: an α/β combination or a γ/δ heterodimer[38] . Over 95% of T cells express an α/β TCR. The *α* and beta chains each have a variable domain and a constant domain. The variable domain is what defines the TCR's antigen specificity and is at the N-terminal domain of the receptor (facing outwards). The antigen specificity is determined by a predefined

set of genes called V, D and J-genes. The extensive repertoire of VDJ-genes and random V(D)J recombination generates a potential repertoire of up to 10^{15} unique receptors (**Figure 3B-C**)[43] ! The beta-chain consists of VDJ-genes but the α chain only contains VJ-genes. This recombination process is mediated by the RAG1 and RAG2 enzymes which recognize a signaling element flanking the VDJ genes (recombination signaling segment, RSS), introducing double strand breaks which excise the other VDJ genes, leaving only one V, D and J gene respectively fused together[44,45] . A constant domain downstream of the VDJ region (or VJ for the α chain) anchors the TCR to the cell membrane[38] .

The antigen-binding site is formed by complementary determining regions (CDRs), short amino acid segments encoded by the V, D, and J genes. While non-CDR amino acids in these genes do not bind the antigen epitope directly, they are important for correct folding of the tertiary structure of the CDRs and the variable domain overall. Overall the CDR has much greater variability in their amino acid sequence, and are therefore referred to as *hypervariable regions*[46] . There are three CDRs (CDR1-3) and of these CDR3 is the primary responsible for antigen-peptide recognition and is generated by both the V and J genes (for α chain) and D and J genes (for Beta chain). CDR1-2 are in turn encoded by the V gene and these regions recognize the MHC molecule itself[47] . Since the CDR3 is crucial for antigen-binding, the genetic sequence differs greatly between T-cells from different clones and is used in research to classify T-cell clones using advanced sequencing techniques (next-generation sequencing for instance). This is important for vaccine research since the information provided by analyzing the CDR3 can help understand which epitopes generate a robust T-cell response and immune memory[48,49] . Studying T cell responses to foreign antigens (e.g., the SARS-CoV-2 spike protein) may enable the design of vaccines containing peptides that induce a strong and protective immune response.

While the α and β chains of the TCR are sufficient for MHC-peptide binding, they cannot independently generate intracellular signaling to activate the T cell[50] . The TCR associates with the CD3 co-receptor, a protein complex of four chains (γ, δ, ε, and ζ), which compensates for the TCR's lack of intracellular signaling domains. The CD3 complex compensates for the TCRs lack of intracellular signaling ability (**Figure 3C**). Once the TCR binds to its antigen-peptide complex on MHC, the CD4 or CD8 co-receptor activates the Lck kinase, which phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) present in the CD3 chains[38] . Phosphorylation of these ITAMs enables ZAP-70 (ζ-chain-associated protein kinase 70) to further phosphorylate intracellular proteins, initiating a signaling cascade that activates the T cell to secrete cytokines, proliferate, differentiate, and survive[51]. However, despite the intricate process

described above leading to TCR signaling, antigen-presenting cells and T-cells need to be in close proximity physically to have a successful interaction. This interaction forms what is known as the immunological synapse[52] , a specialized junction that facilitates communication between T-cells and antigen-presenting cells.

THEE IMMUNOLOGICAL SYNAPSE

While the interaction between the T-cell receptor (TCR) complex and peptide-MHC complex is crucial for antigen-specific recognition and T-cell activation, it is insufficient to fully activate the T-cell. This limitation is attributed to several factors as elegantly discussed by Grakoui et al [53] and Professor Dustin [59]. First, the T-cell is not a static cell and is moving around in the lymph node, for a proper interaction with the APC they both need to adhere to each other. Secondly, there exists several large glycoproteins which can physically block/impair the interaction between the TCR-MHC complexes. The affinity of TCRs for their peptide-MHC ligands is typically in the micromolar range (1-100 μM), which is relatively low compared to the picomolar to nanomolar affinities of high-affinity antibodies[53,54] . Unlike B cell receptors (BCRs), TCRs do not undergo somatic hypermutation (discussed later) which generates these high-affinity BCRs[55] . The availability of MHC-peptide complexes on a single cell is limited, with estimates suggesting it can be as few as 100 peptide-MHC complexes per cell [56] . How can they overcome these obstacles? Firstly, similarly as the leukocytes bind to the endothelial cells using integrins to adhere to the endothelial wall, the T-cell utilizes the integrin LFA-1. This integrin consists of two subunits, an alpha (CD11a) and a beta subunit (CD18). LFA-1 exists in three conformational states low, intermediate, and high affinity which determine its binding affinity to ligands[57]. These states can be induced by either ligand-binding (outside-in signaling) or by chemokine and TCR intracellular signaling (inside-out signaling)[58]. In the context of T-cell-APC interaction, LFA-1 will undergo a conformational change from low-affinity to temporary high-affinity stage through chemokine signaling (through G-protein coupled receptors) and or TCR complex signaling. Subsequently, LFA-1 binds to ICAM-1/CD54 ligands on the APC, inducing a more stable high-affinity state[59]. These transient interactions are referred to as 'kinapses' while the T-cell remains in motion[60]. Once enough integrins are activated the T-cell will stably adhere to the APC. Moreover, the T-cell extends its cell-membrane in a ring-shape around the MHC-TCR complex. This change has the effect of removing the sterical obstacles of glycoproteins which disrupt the MHC-TCR interaction physically. Additionally, clustering of TCRs within this membrane ring increases the avidity of the interaction, thereby enhancing signaling despite the relatively low affinity of individual TCRs [61]. This has been shown to be LFA-1-ICAM1 mediated[62]. The mature immunological synapse contains distinct clusters of receptors and molecules known as

supramolecular activation clusters (SMACs)[52]. These clusters include a central cluster containing TCR complexes with its co-stimulatory molecules such as CD28 (central supramolecular activation clusters, cSMAC)[63], a peripheral ring of LFA-1 enriched proteins (pSMAC) and a distal cluster containing CD43 and CD45 (dSMAC)[64]. The segregation of the different molecules in the dSMAC and cSMAC is crucial. CD28 helps amplify and prolong T-cell activation, proliferation and survival (through costimulatory intracellular signals) in conjunction with TCR signaling. While CD45 is a phosphatase and its removal from the cSMAC shifts the dynamic towards kinase activity (allowing for phosphorylation of CD3)[59]. Furthermore, in the dSMAC, CD45 activates Lck (a kinase) from its inactive state which can further enhance downstream TCR signaling. The composition and spatial position of the different SMACs are essential for allowing TCRs to activate the T-cell despite the relatively low-affinity nature of TCRs (compared to antigen-experienced BCRs). Taken together, the immunological synapse is a crucial structure for T-cell activation upon recognition of antigen-peptide complexes on MHC molecules. Protein-protein interactions, clustering effects to amplify intracellular signaling, and enhanced avidity are all essential, multi-step processes for robust immune activation. The concept of how clustering of receptors and their ligands in the immunological synapse leads to T-cell activation, will be important for future chapters (**Chapter 2, 5** and Paper I-III particularly) when we discuss antibody function by activating effector cells.

T-CELL ACTIVATION AND FUNCTION

Traditionally the model for T-cell activation by APCs is described as needing two signals, one which is induced by the TCR-MHC complex interaction and the second signal is cumulative intracellular signaling by co-stimulatory receptors CD28, CD40L and LFA1 to their ligands on the APC (CD80/86, CD40 and ICAM-1 respectively)[65]. The activation process varies depending on whether the T-cell expresses CD4 (naive CD4+ cells) or CD8 (naive CD8+ cells), since the double positive status of T lymphocytes is lost when encountering APCs in the secondary lymphoid organs. CD4+ T-cells are referred to as T helper cells (note:regulatory T-cells also express CD4), since they are responsible for activating the CD8+ Cytotoxic T-cells and B-cells later. Upon receiving signal 1 (from MHC-2) and 2 by APCs, the naive CD4+ cell which recognizes the foreign antigen of interest will survive, proliferate (clonal expansion) and differentiate (influenced by cytokines)[66]. Similarly, once the CD8+ cell recognizes its MHC-peptide antigen complex, CD4+ T-cells will help activate them by producing cytokines vital for survival and proliferation[66].

Helper T-cells coordinate the immune response for more effective pathogen elimination, with distinct subsets arising upon differentiation[65]. Th1 cells are potent

producers of interferon-γ, IL-2 and TNF-α which enhances macrophage phagocytosis, ROS production, MHC-2 expression and upregulation of costimulatory receptors thereby improving macrophages' antigen-presenting capabilities. All aforementioned enhanced functions by macrophages due to Th1 cytokine release enhances immune defense against viruses and bacteria (intra-and extracellular) [65]. Th2 cells are classically deemed to be skewed in immune responses against helminth parasites and in allergy. A subset of T-cells expressing CD4 are known as Treg cells and they eliminate autoreactive T-cells which recognize self-antigen (which have escaped elimination in the negative selection process in the thymus) [65]. Finally, as we mentioned before, CD8+ cells are important for eliminating virus-infected cells which display foreign antigens on MHC-1. A subset of T cells (both CD4+ and CD8+) differentiate into long-lived memory cells that can rapidly expand upon re-infection with the same pathogen (or a pathogen with cross-reactive epitopes). Follicular helper T (TFH) cells are important for upregulating B cell antibody production and B cell activation, as discussed in the subsequent B cell section.

B-CELLS

Like T-cells, B-cells originate from a common lymphocyte progenitor in the bone marrow before immature B-cells migrate to secondary lymphoid organs such as lymph nodes and the spleen[67]. Prior to engaging with antigens and follicular T-cells in these distant organs, immature B-cells undergo changes in their receptor expression. For a proper activation by T-cells, B-cells need to acquire its co-receptors such as CD20, CD21, and CD40 which all play an important role in intracellular signaling leading to survival, differentiation and clonal expansion (more on this later). Immature B-cells leaving the bone marrow will express a BCR which has undergone similar V(D)J recombination by RAG1/2 enzymes in a similar process leading to the generation of the TCR as we discussed before[44,45]. Similarly as with the TCR, there exists an extensive repertoire of VDJ-genes, and the different combinations generated by the double strand DNA breaks by RAG1/2 can produce more than 10^{18} unique BCRs[68]. The B-cell receptor (BCR) consists of four polypeptide chains: two identical heavy chains and two identical light chains. Both the light and heavy chains consist of a variable domain and a constant domain. The variable domain is made up of the V(D)J genes. There are several constant domain genes for the heavy chain, including IGHD, IGHM, IGHG, IGHE, and IGHA. In the immature B-cell and the first BCRs generated in the bone marrow, the maturing B-cell utilizes primarily the IGHM locus for its initial BCR[37] . When the immature B-cell leaves the bone marrow, it co-expresses two receptors, one containing the

IGHD gene in the heavy chains and one in the IGHM gene. On the light chain side, the variable domain will be attached with either a kappa or lambda constant domain. The naive B-cell will thus express a fully assembled BCR utilizing the IGHD and IGHM gene locus when encountering the APC and follicular T-cells in the secondary lymphoid organs, and upon antigen recognition will survive, proliferate and expand. Before exiting the bone marrow, BCRs undergo testing for self-antigen recognition through positive and negative selection processes, and the BCRs that fail to pass these tests will be eliminated by cell apoptosis[69,70]. Alternatively, if a BCR fails these tests, it can undergo receptor editing through reactivation of RAG1/2 enzymes. This is a crucial step since self-reaction can lead to autoimmune disease with detrimental host-effects, such as multiple sclerosis where antibodies bind to myelin on neurons which is thought to occur due to Epstein-barr virus infection, a process called molecular mimicry (when foreign substance share similarity with host leading to cross-reactivity)[71,72] .

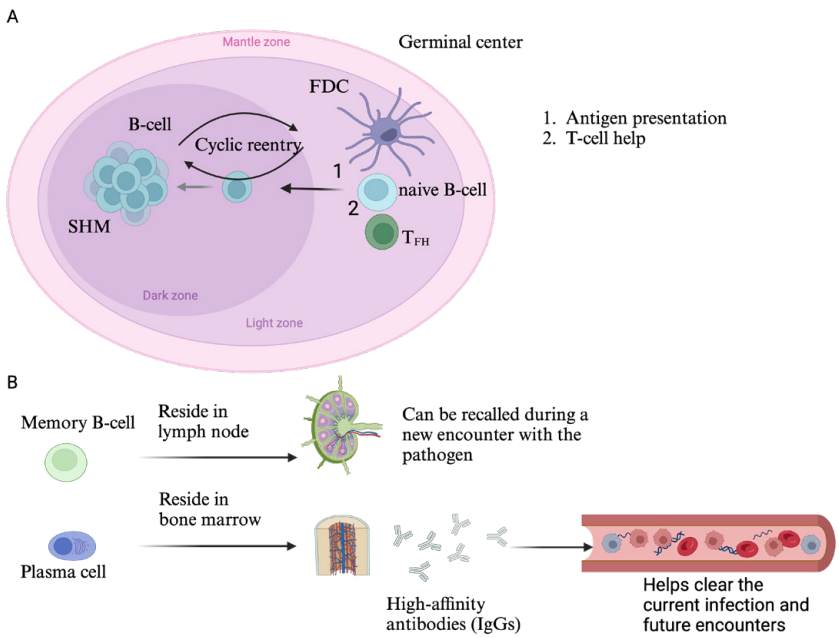

Figure 4. Fate of B-cells in the germinal centers A Illustration of the structure of the germinal center, in a light zone, dark zone and mantle zone. B-cell activation occurs in the light zone by Follicular dendritic cells (FDCs) and T follicular helper cells (TFH), and affinity maturation occurs in the dark zone. B-cells can re-enter the dark zone for further rounds of mutations (called cyclic reentry). In figure **B** shows how successful B-cells can either become memory-B cells or plasma cells. These cells have different roles for long term immunity and reside in different immune compartments. Made using biorender.com. Note only ”surviving” B-cell clones are depicted, a majority of clones do not proliferate and differentiate.

B-CELL ACTIVATION

Naive B-cells upregulate chemokine receptors and migrate via chemotaxis to the lymph nodes and enter the B-cell follicles, where the B-cells will later expand upon antigen-encounter and specialized structures called germinal centers (GCs)[39] . The germinal center is organized into two distinct zones: a light zone and a dark zone, where the former houses both follicular helper T-cells (T_{FH}) and APCs (follicular dendritic cells, FDCs) (**Figure 4A**)[73,74] . FDCs attract naive B-cells through the secretion of the chemokine CXCL13. FDCs lack the ability to phagocytose the antigen immune complex, the antigen is therefore unprocessed and not presented as digested peptides to B-cells (as in the case of T-cells)[75]. The antigens will be displayed as immune complexes, by being bound by "natural opsonins" such as antibodies and complement protein (C3). The antibody-antigen complex will bind to CD32b (which is a low-affinity Fc-receptor for the IgG class of antibodies, Fc-) on FDCs[73] . Furthermore, the complement-deposited antigen complex will be bound by complement receptor 1 and 2 (CR1-2), CD21 and CD35 respectively. The FDCs can store these antigens without processing them for a long time in endosomal compartments, with great importance for increasing antibody affinity (as we will discuss later)[76] . When encountering the FDCs antigen-complex, the corresponding BCR will initiate binding to the antigen. Currently, there is no consensus on a unifying model for how BCRs are triggered; three different models have been proposed (see Degn & Tolar review)[77] . Irrespective of the exact process of how BCRs are triggered, the defining interaction is that of the antigen displayed on APCs with the BCR. Moreover, the B-cell receptor complex contains two co-receptors in addition to the BCR, CD79 α and CD79β, which play a similar role as CD3 for TCRs where they enable phosphorylation of their ITAM motifs by intracellular kinases. Phosphorylation of ITAM motifs triggers an intracellular signaling cascade that facilitates APC-B-cell adhesion. The adhesion process is mediated through LFA-1 interaction with ICAM-1 on APC but also VLA4(CD49d/CD29) to VCAM-1 (CD106), cytoskeleton rearrangement allowing formation of the immunological synapse and activation of transcription factors important for survival and proliferation[78] . CD19 and 21 are two co-receptors with the important function of enhancing the BCRs signaling through inhibiting degradation of the receptor, enabling prolonged signaling[79] . BCRs can recognize soluble antigens; however, this interaction typically results in weaker B-cell activation compared to interactions involving clustered antigens in the cSMAC. The clustering of BCRs and antigens increases the avidity and allows even low-affinity interactions to generate a B-cell activation. This highlights the significance of the immunological synapse in enhancing antigen recognition through avidity increases by clustering of BCR-antigen complexes in the cSMAC (believed to be through cytoskeleton

rearrangement similarly as with T-cells)[80]. Increasing avidity through clustering allows for greater antigen recognition through BCR signaling of even lower-affinity interactions (reportedly in the microM affinity range)[81]. The low affinity of the BCR to the antigen can be further improved during *somatic hypermutation* (which can enhance affinities several orders) which occurs after B-cell survival and during proliferation in the dark zone of the germinal centers (more on this later).

While TCR activation leads to proliferation and survival, the BCR signaling has a dual-role where it also needs to extract the antigen physically from the APC to later display it to the follicular helper cells on MHC-II complexes [82,83]. BCR signaling facilitates colocalization of the actin cytoskeleton with cSMAC BCR-antigen clusters and by the help of myosin contract so the antigen is pinched off from the APC[84]. Different APCs exhibit varying membrane stiffness; for example, FDCs have a higher threshold for antigen release compared to DCs[82]. Thus the bar for how low affinity the BCR can have to its antigen is raised since a weaker affinity-antigen interaction would not be able to withstand too much mechanical force (thus removing the BCRs with weak affinity). The BCR-antigen complex will subsequently be internalized in the cell in the form of an endosome. Thereafter, the endosome will be fused with a lysosome which will degrade the antigen to peptide segments that will be reallocated to the cell membrane on MHC-1 molecules. Follicular T-cells with TCRs specific for this antigen will recognize it presented on MHC class II molecules. Furthermore, the CD40 ligand (CD40L) on the T_{FH} in addition to cytokines and CD28 (binding to B7 on B-cells) will promote survival (through upregulation of anti-apoptotic transcription factors such as BCL-2), proliferation and differentiation by the now naive turned activated B-cell[54]. The activated B-cell will then mature and proceed to either 3 different fates: become a short-lived plasma cell which secretes antibodies (the BCR), become a memory B-cell (independent of GC maturation) or enter the GC dark zone and undergo somatic hypermutation to alter affinity of the BCR to the antigen and then engage again with the FDCs for another cycle (*cyclic reentry*) of BCR-testing (a process called *affinity maturation*) (**Figure 4A-B)**. In addition to these fates, activated B-cells can differentiate into plasmablasts, which are rapidly dividing antibody-secreting cells[54]. These cells serve to produce the initial wave of antibody production which combats the infection in an early stage of the adaptive response. A subpopulation of these plasmablasts can further differentiate into long-lived plasma cells. Plasmablasts can be viewed as an intermediate step between these long-lived plasma cells and the activated B-cell. These cells migrate to the bone marrow (but can also migrate to the spleen and mucosal tissue) and continue to secrete high-affinity antibodies for extended periods, contributing to long-term humoral immunity (hence their name) (**Figure 4B)**. The T-cells are heavily involved in determining

which pathway the activated B-cell undergoes, where for instance the transcription factor MYC, induced by T_{FH}, is tied to differentiation into plasma cells and not memory B-cells[86].

It is worth noting that BCRs can be activated independent of T_{FH} and FDCs by directly engaging with the pathogen through PPRs, this will not lead to T_{FH} engagement since the BCR will not internalize the antigen. The T-cell independent activation by these B-cells allow for a fast and innate-immune-like initial response. These B1 B-cells differentiate into short-lived plasma cells that secrete IgM antibodies, which are crucial for immediate defense against pathogens but typically lack the affinity maturation seen in conventional B2 cells. These antibodies, due to the lack of SHM, can have lower affinity to their antigens[37]. However it is entirely possible that germline antibodies (without SHM which have only undergone VDJ-recombination) can have nanomolar affinity to epitopes[87].

Class-switch recombination

While the variable domain of the B-cell receptor (BCR) is crucial for receptor activation, the constant domain significantly influences the effector functions of antibodies. After BCR activation, B-cells undergo class-switch recombination (CSR) where the constant domain of the receptor can be switched from IGHD and IGHM to IGHG, IGHA or IGHE[88] . Immunoglobulins consist of several classes and subclasses, each determined by the constant domain of the heavy chain. It was previously believed that CSR occurred in the dark zone; however, recent findings indicate that CSR takes place in the light zone following BCR activation[88]. The CSR process is heavily influenced by T-cell signaling through CD40 ligand (CD40L). In previous work various cytokines have been implicated in modulating the expression of different classes of antibodies. In humans for instance, IL-4 secretion by Th2 cells induces a switch to IgG4 and IgE antibodies, while IgG3 and IgG1 can be induced by IL-10 by Th1 cells and IL-21 from T_{FH} cells[89].

The process of CSR depends on an enzyme called Activation-induced Cytidine Deaminase (AID) which initiates the excision of the exons proceeding the gene of interest[90]. AID expression is upregulated by cytokines such as IL-21 and by B and T-cell interaction (through CD40-CD40L)[91]. BCR-antigen engagement also increases AID expression. AID induces both single and double-strand breaks for this to occur in the conserved region upstream of the immunoglobulin constant domain genes. Immunoglobulin constant domain genes are organized downstream of the variable gene segments and follow a specific order: IgM, IgD, IgG (IgG3, IgG1, IgG2 and IgG4), IgA (IgA1-2) and IgE. For example, if a BCR switches to

an IgG3 constant domain, the upstream IgM and IgD exons will be excised through AID activity.This excision results in double-strand breaks that allow downstream variable genes to be joined with the IgG3 constant domain gene through non-homologous end joining. Important to note, once a constant domain locus has been excised the B-cell cannot express that particular class or subclass of antibody anymore. The B-cell which has undergone CSR will either become a GC-independent memory B-cell, a short-lived plasma cell which secretes this new IgG3 antibody (in our example) or enter the dark zone for further BCR editing via somatic hypermutation, an important process for affinity maturation; these cells are typically referred to as GC-dependent B-cells)[37].

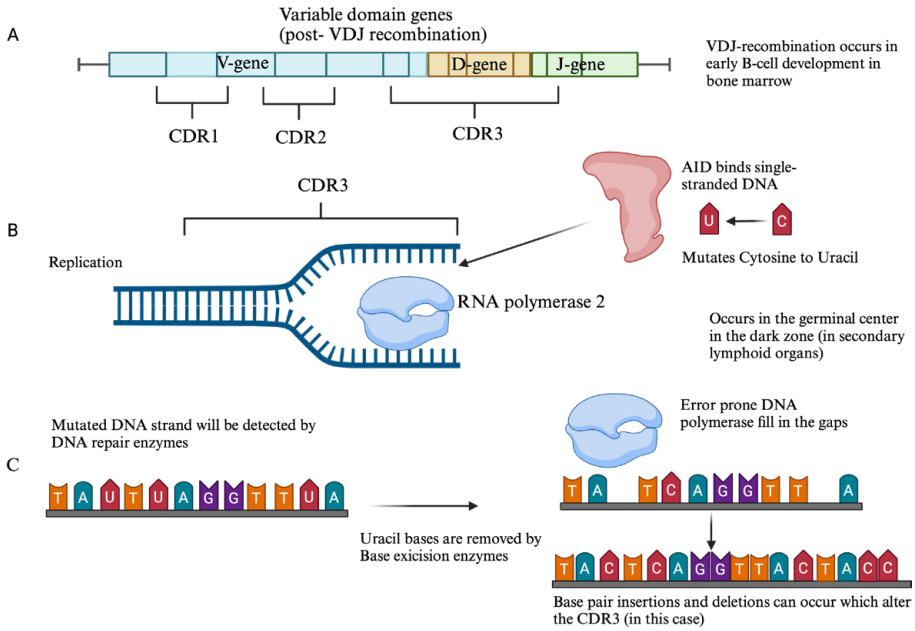

Figure 5. Somatic hypermutation in the germinal center A Illustrates the CDR domains in the VDJ, where the CDR3 is more diverse, being composed of the V, D and J- gene respectively. Somatic hypermutation is induced by AID enzyme activity which induces a mutation by changing Cytosine to Uracil which triggers repair mechanisms which are more error-prone than in other cell-lines leading to permanent mutations as illustrated in **B-C.** Made using biorender.com**.**

SOMATIC HYPERMUTATION

While GC-independent B-cells possess antigen specificity, their BCRs typically exhibit weaker affinities compared to those selected by follicular dendritic cells (FDCs) after undergoing somatic hypermutation (SHM) in the dark zone[55]. In one

study, focusing on BCRs against a known HIV antigen, the authors showed that the BCR affinity towards the HIV antigen of interest was in the micromolar range[78]. Contrary to those B-cells, the memory B-cells which have undergone SHM had affinity of up to 100-fold, enhancing their ability to activate the B-cell and internalize the antigen. The process of SHM has been shown to be dependent on the enzyme AID (**Figure 5A**). Due to the increased mutational rate in the variable genes (up to 10^6 times compared to other genes) it is vital that only these genes are targeted by the AID enzyme to avoid inducing B-cell malignancies[92]. It has been shown that there exists a hot-spot for mutational frequencies in the variable genes, particularly around 150 base-pairs downstream of the transcriptional promoter of the variable genes to 1-2 thousand base pairs. The constant domain of the BCR is not affected by SHM. It is not entirely established how AID induces mutations, but a model which has been proposed is that during transcription, the double stranded DNA will be unwinded to single-stranded DNA, which will enable binding of a transcription factor called Replication Protein A (RPA)[93]. RPA binds to single-stranded DNA and facilitates AID's deamination of cytosine into uracil (**Figure 5B**). From there, the U-G mismatch will activate several enzyme systems such as DNA polymerase (which will convert the U-G to a T-A instead). Base-excision repair enzymes remove uracil from U-G mismatches, while mismatch repair enzymes (MMR) may introduce additional mutations or insert A-T nucleotide pairs (**Figure 5C)**. In summary, the SHM process is unique to the BCR, with variable domain genes serving as hotspots for AID activity, leading to mutations that may enhance affinity. The variable domain genes are a hotspot for the activity of AID to insert mutations which due to the activity of more error-prone repair enzymes creates new sequences with potentially stronger affinities (a clear majority of mutations are not affinity-enhancing). After undergoing SHM in the dark zone, these BCRs re-engage with FDCs in the light zone to test their new affinities; this feedback mechanism is commonly referred to as *affinity maturation.*

AFFINITY MATURATION

After exiting the dark zone, activated germinal center (GC)-dependent B-cell clones undergo proliferation and diversification into subclones, with varying BCRs as a result of SHM (which occurred in the DZ)[94] . Through the process of cyclic reentry they will migrate towards the light zone and engage the FDCs with their BCRs. The GC B-cell differs in several important aspects compared to the naive B-cell. Firstly, it has been suggested that the naive B-cell is less dependent on T_{FH} cells for survival due to tonic signaling of the BCRs which promotes survival. For GC B-cells, they are more dependent on CD40-CD40L and paracrine cytokine release by T_{FH} cells to survive and further differentiate [95]. Secondly, the affinity of the BCR to the

displayed antigen differs from the naive B-cells due to SHM. While the FDCs have higher stiffness and require stronger mechanical pull (ability to internalize antigens displayed by FDCs) than other antigen-presenting cells, as discussed previously, evidence suggests that the GC B-cell exerts a stronger mechanical pull than the naive B-cell once bound to the antigen[80,82]. In addition, while the BCR-antigen complex is centralized in the immunological synapse by the naive B-cell, the GC B-cell has been shown to push the antigen into peripheral clusters of the immunological synapse. These factors generate an even greater constraint on the BCR-antigen binding strength and thus select for higher-affinity BCRs (which successfully internalize more antigen)[39]. It has been theorized that the BCR affinity to antigen correlates with the amount internalized and later displayed to the TCRs on MHC-complexes[96] . The overall strength of the engagement by T_{FH} and GC B-cells influences diverse transcription factors which determine the GC B-cells fate. It has been suggested that high-affinity BCRs will promote stronger T_{FH} help (cytokine secretion), and push cell-fate towards becoming high-affinity antibody producing long-lived plasma cells[37,54,94] . While intermediate T_{FH} engagement will promote cells to become GC B-cells which stay in the germinal centers and low engagers will drive the cell to become a lower affinity memory B-cell. Furthermore, a GC B-cell can reenter the dark zone for further rounds of SHM as part of the cyclic reentry. Of course many B-cells have been producing defective BCRs and not high-affinity ones, and they are eliminated through apoptosis. The interaction with T_{FH} is dependent on several variables such as BCR affinity to antigen, TCR affinity to antigen, density of antigens presented by the FDCs and cytokine expression by the T-cells (IL-21 for instance which induces AID expression leading to more SHM). Taken together while much is unknown regarding the processes occurring in the GCs, more evidence has emerged showing that the affinities of the BCRs correlate with their differentiation into becoming high-affinity long-lived plasma cells or memory B-cells (independent and dependent of the T-cell help respectively) with lower affinities.

Depth and breadth, generating a specific immune response but allowing flexibility for future adversaries

Recent technological advances in single-cell sequencing have enabled scientists to study the B cell receptor (BCR) repertoire following infection and vaccination. What has been shown is that, generally, B-cells which become long-lived plasma cells (through strong T_{FH} help) have increased affinity for its epitope compared corresponding memory B-cells[37,54]. These cells are responsible for antibody production and secretion, thus providing a strong presence of anti-pathogen antibodies in blood and mucosal areas. The high-affinity binding of these antibodies

to their antigens (reflecting the BCR-affinity as discussed above) is a precise attack at the pathogen, where a future encounter will enable swift elimination (generally). However, what if a pathogen undergoes mutational changes in the epitopes targeted by long-lived plasma cells? Or what if an emerging pathogen, belonging to the same family as a previous one, presents differences in the antigen of interest (such as SARS-CoV-2 variants)? In these scenarios, the lower-affinity BCRs of memory B cells become valuable. It is in this context where the lower-affinity BCRs of the memory cells are put to good use. Their less stringent specificity allows for broader epitope recognition. The memory B-cells will be recalled and re-enter the lymph nodes where they will recognize the mutated pathogens protein (or new pathogen with similar protein) on the FDCs. These memory B-cells who recognize the foreign antigen can be selected to undergo SHM and CSR and become long-lived plasma cells capable of producing high-affinity antibodies specific for the new threat. Of course, naive B cells can also mount a novel response against this new threat. Therefore, low-affinity antibodies shouldn't be discounted, as they provide versatility in recognizing emerging pathogens or mutated variants, allowing time for a new, adapted immune response to develop. The existence of plasma cells and memory B cells thus provides both depth and breadth in the humoral immune response. The next section will explore the biology of antibodies in greater detail.

Author comment on the chapter:

Having discussed the key players in the immune response to bacterial and viral pathogens, the next chapter will focus on the biology of antibodies. However, it is worth noting that, despite belonging to different subfields of Immunology, there are notable similarities in how T-cells function and are activated by dendritic cells, and how antibodies activate effector cells (such as the formation of the phagocytosis cup). In the immunological synapse, avidity interactions (the clustering of receptors with ligands) and biophysical factors, such as the segregation of inhibitory proteins, are essential for efficient activation. Similarly, the binding of a single antibody to its antigen does not trigger sufficient activation or response. As we will explore, antibodies are highly dynamic proteins that require cooperation to fully activate Fc-mediated effector functions, such as phagocytosis, complement activation, and antibody-dependent cellular cytotoxicity. However, theory does not always align with real-world experimental data, so it is crucial to maintain an open mind, because sometimes the theory needs revision. As we will discuss in the opening section of the next chapter, the classical dogma that antibody variable and constant domains are independent and do not influence each other has faced a lot of

challenges, of which findings in Paper 1 and 2 also contributed. As Einstein once said, 'The more I learn, the more I realize how much I don't know.'"

CHAPTER II
ANTIBODIES AND THEIR FC-RECEPTORS

A MODEL CHALLENGED- VARIABLE AND CONSTANT DOMAIN DEPENDENCE?

Human antibodies are Y-shaped proteins which consist of two identical heavy and light chains. The variable domain is unique to each antibody, generated via random V(D)J recombination and further diversified by somatic hypermutation (SHM)[97]. The variable domain contains complementarity-determining regions (CDRs) that determine antigen specificity. In contrast, the constant domain is encoded by a fixed set of genes, which are neither randomly combined nor mutated during SHM. There are five families of genes encoding the constant domain: IgD, IgM, IgG, IgA, and IgE, with IgG and IgA containing four and two genes, respectively. The constant domain is divided into subdomains: CH1 (present in the Fab domain, adjacent to the variable domain), CH2, and CH3 (constituting the Fc domain). The Fab domain (fragment antigen-binding) is formed by the entire light chain (variable and constant domains) and parts of the heavy chain (variable domain and CH1). The Fab and Fc domains are linked by a hinge region, encoded by the constant domain (**Figure 6A**).

Although the structure of antibodies is well-characterized, recent research challenges the traditional view of independent variable and constant domain function. Historically, it was believed that the constant domain did not affect the variable domain's antigen-binding, and conversely, the variable domain did not influence FcR interactions or other Fc-mediated activities[98]. In other words, the constant domain was thought to have no effect on the variable domain's binding to its antigen, nor did the variable domain influence Fc-FcR interactions or other Fc-mediated activities. This view has been challenged by both older and more recent observations, revealing a more nuanced picture where the constant domain influences the binding-properties of the variable domain, and the variable domain influences the constant domain's ability to interact with FcR[99]. Research has shown that changes in the constant domain can enhance or reduce the affinity of the variable domain for its antigen[100–102], indicating a more integrated mechanism of action than previously thought. When studying the importance of constant domain for antibody function, researchers have engineered monoclonal antibodies to express different constant domains (while not altering the variable domain). These studies showed that antigen-binding affinity was increased or decreased, suggesting

that the constant domain can indeed influence how the variable domain engages the antigen[100–102]. In summary, antibodies are highly dynamic proteins, and a revised model is needed to understand their function, accounting for the interplay between variable and constant domains. Class-switch recombination adds further diversity to both the functional and binding properties of antibodies, and future research should investigate these mechanisms. This thesis will address this issue in greater detail, particularly in the discussion of papers 1 and 2.

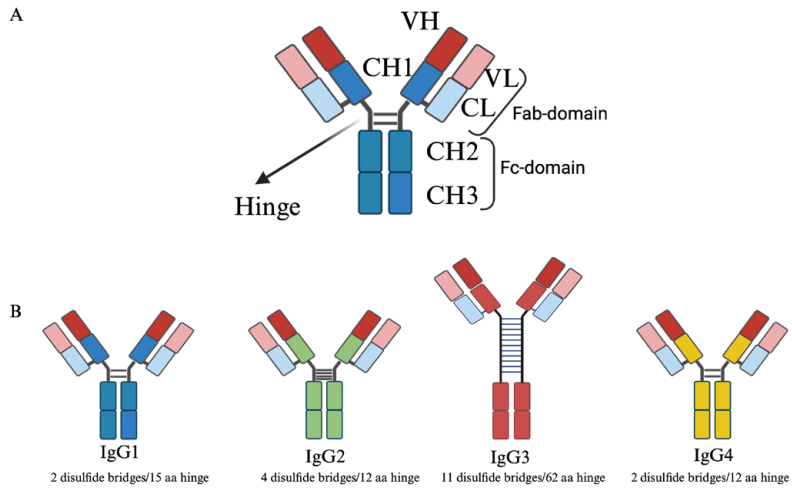

Figure 6. Schematic overview of the different domains of IgG antibodies. A Illustration depicting the Fab and Fc domains, highlighting the respective subdomains within. The Fab domain consists of the entire light chain (variable and constant domains), the variable domain of the heavy chain, and the constant domain 1 of the heavy chain. The Fab domain is linked to the Fc domain by the hinge region. The Fc domain consists of constant domains 2 and 3. Note: Figure **B** illustrates the differences in hinge length and the number of disulfide bonds in the hinge for the four IgG subclasses. For IgG3, one common allotype with a 62-amino acid hinge is depicted. Created with BioRender.

The Immunoglobulin G Subclasses - An Overview

While IgD and IgM are present initially in the infection, upon naive B cell activation in the GC, B cells can undergo CSR to encode a BCR with an IgG constant domain. The IgG family consists of four subclasses encoded by separate genes: IgG1, IgG2, IgG3, and IgG4[97]. These genes encode three subdomains, designated constant domains 1, 2, and 3 (as discussed previously). The Fab and Fc domains are linked

by a hinge region, also encoded by the constant domain exon, spanning from the end of CH1 to the beginning of CH2 (**Figure 6A-B**).

Of particular interest to this thesis, the hinge region of IgG3 is longer than those of IgG1, IgG2, and IgG4. IgG1 has a hinge region 15 amino acids long, while IgG2 and IgG4 each have a 12-amino acid hinge region. Several different allotypes of IgG3 exist, but the minimum number of amino acids in the IgG3 hinge is 32, and the maximum and most common is 62 amino acids [97]. Hinge length is known to influence IgG antibody flexibility. Additionally, inter-hinge disulfide bridges are associated with rigidity/flexibility, with the IgG2 subclass containing up to 4 inter-chain disulfide bridges, making it more rigid than IgG1 and IgG4 (which have two). IgG3, depending on its allotype, has between 11 and 15 disulfide bridges in its hinge region. Hinge length significantly influences antibody flexibility, with the order being IgG3 > IgG1 > IgG4 > IgG2. The evidence for this order of flexibility originates from a well-cited study in the late 1990s by Roux and colleagues[103] . This study used electron microscopy to examine the flexibility of different IgG subclasses in solution. They defined flexibility by both the angle between the Fab arms (Fab-Fab angle) and the angle between the Fab and Fc domains (Fab-Fc angle). The authors reported differences, measured as the standard deviation of the angle, in the order of a few degrees: IgG3 (±36°) > IgG2 (±32°) =/> IgG1 (±30°) > IgG4 (±25°). These numbers reflect only the calculated smallest Fab-Fc angles, since there are two Fabs, and a large Fab-Fc angle for one Fab arm correlates with a smaller Fab-Fc angle in the other arm. Therefore, studying the overall Fab-Fc angle might lead to angles that cancel each other out when averaging. Similarly, flexibility measured as a Fab-Fab angle was consistent with the order of flexibility: IgG3 (±52°) > IgG1 (±43°) > IgG4 (±39°) > IgG2 (±32°). When discussing antibody flexibility, it is important to specify the type of flexibility: that of the Fab arms relative to each other, or that of the Fab arms relative to the Fc domain. This distinction has important implications, as increased Fab-Fab flexibility might facilitate cross-linking of antigens while altered Fab-Fc flexibility might modulate interactions with FcRs for efficient clustering. Additionally, non-Fc functions such as neutralization can be modulated by manipulating the upper hinge region connecting both Fabs[104] . As shown in Paper II of this thesis, this is a complex issue, and nuance is critical when discussing antibody flexibility.

Significant functional differences exist between the IgG subclasses. Specifically, IgG1 and IgG3 are classically described as being more pro-inflammatory because they have higher affinity for the Fc-γ receptors (CD64, CD32, and CD16) [97]. Additionally, they activate the classical complement pathway much more efficiently than IgG2, while IgG4 cannot activate it at all. These functional differences are

attributed to amino acids in constant domain 2, the subdomain that engages with FcRs and the complement protein C1q (which assembles the C1 complex, initiating the classical complement pathway) [97].

Furthermore, a glycosylation site within the CH2 domain is heavily involved in FcR engagement, making glycoengineering (altering glycans in the antibody) a popular approach to modulate monoclonal antibody function (discussed further in the next chapter) [97]. Lower in the CH3 domain, amino acid motifs bind to the human neonatal Fc receptor (FcRn), which recycles human IgG upon internalization in the cell, influencing IgG half-life in blood.Human IgG3 contains an arginine instead of a histidine at residue 435, which shortens its half-life to 7 days compared to 21 days for IgG1, IgG2, and IgG4. Interestingly, allotypes of IgG3 exist with the H435 variant, and these antibodies have half-lives like those of the other subclasses[105] . For more details on IgG subclasses, please find the excellent review by Vidarsson et al [97] . Understanding these subclasses is crucial for natural immunity and post-vaccination responses against various pathogens. In subsequent sections, we will explore the affinities of these subclasses for human Fc-γ receptors.

ANTBODIES AND THEIR FC-RECEPTORS- IT TAKES TWO TO DANCE

As described previously, the molecular affinities of human Fc-γ receptors vary depending on the IgG subclass and the specific receptor. IgG1 and IgG3 have higher affinity for CD64, CD32, and CD16 compared to IgG2 and IgG4 (the latter cannot bind to CD64, has moderate affinity for CD32, and only weakly binds to CD16)[106,107]. Affinity is quantified by the dissociation constant (KD), an equilibrium constant that reflects the ratio of bound (antibody-antigen complex) to unbound antibody and antigen. Therefore, a high concentration of unbound antibody and antigen indicates a high KD, reflecting lower affinity (as the antibody-antigen complex dissociates rapidly). KD is typically expressed in molar units[108] . A lower K_D for an antibody to a FcR is therefore a high-affinity binding, where nanomolar K_D values are classified as high and micromolar (10^{-6}M) is classified as low-affinity binding. However, only CD64 can be efficiently activated by monomeric IgG, i.e., without forming an immune complex with the antigen. CD64 is therefore commonly referred to as the high-affinity Fc-γ receptor, with K_D value in the 10^{-8} to 10^{-10} molar (M) for IgG1 and IgG3. IgG1 and IgG3 exhibit micromolar affinities to CD16 and 32 (10^{-6}M) [97 109] .

In addition to subclass differences, allelic variants of human Fc receptors can also influence binding affinities. It is worth noting that there are allelic variants of human FcR which have slightly higher or lower affinities for the human IgG subclasses,

such as the H131 variant of CD32 (a histidine in the place of an arginine) which binds better to IgG2 for instance[110]. Similarly, another example is CD16a which has an allelic variant V158 which has been shown to be a high-affinity receptor to IgG3 since it can be activated by monomeric IgG3. Furthermore, allotypes of human IgG subclasses can also influence the affinities for Fc-receptors, which is particularly true for human IgG3 which has more allotypes than the rest combined [97] .

There are three subfamilies of CD32 (CD32a, CD32b, CD32c) and two subtypes of CD16 (CD16a, CD16b) [97]. Among these, CD32b serves as an inhibitory receptor, while CD32a promotes immune activation. CD16a/b are activating receptors that mediate effector functions such as ADCC. Differential expression of these receptors on immune cells modulates their responses; for example, myeloid phagocytes express high levels of CD64, whereas NK cells predominantly express CD16a. These cells also express CD32a/b and CD16a (and some have CD16b)[111] . While neutrophils only express small amounts of CD64 (but can be upregulated by interferon-γ release) but mainly CD16a/b and CD32a which allows the cells to exert ADCC and ADCP. NK-cells only express CD16a and some CD32c and mediate only ADCC [111] .

The pre-existing affinities of different IgG subclasses for their respective receptors significantly influence their ability to induce effector functions in these cells [111]. For example, IgG1 and IgG3 are more potent inducers of ADCC and ADCP because they have higher affinities for CD16a/b compared to IgG2 and IgG4. However, it has been shown that the affinity of antibodies to Fc-receptors is also modulated by the formation of an immune complex, the valency of the immune complex (the number of antibody molecules bound to an antigen) has been linked to increased binding and activation[112] . Efficient activation and binding of lower-affinity FcRs depend significantly on the size and valency of the immune complex [112]. This is because the high concentration of IgG in the blood necessitates a high activation threshold, which minimizes unwanted immune activation that could be detrimental to the host [112]. However, a key question is what determines the differences in affinity among these FcRs? This question will be addressed in the section below, focusing on the molecular mechanisms which have implications for Paper II and IV in this thesis.

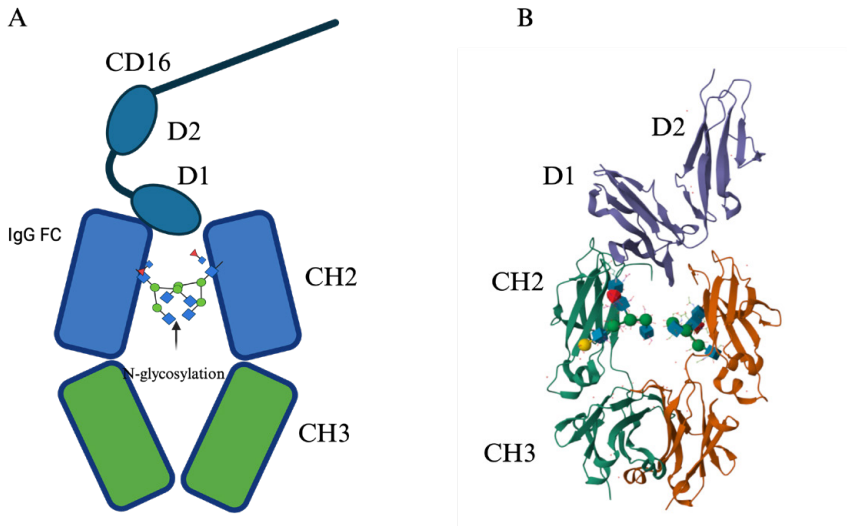

Figure 7. IgG and Fc-receptor binding of CD16. A Depicts CD16 binding to the D1 and D2 domains located in the upper part of the CH2 domain, specifically in the lower hinge region. The glycans in the CH2 domain of the IgG-Fc are illustrated protruding into the horse-shaped cavity "stabilizing" the interaction**. B** illustrates the crystal structure (PDB: 57CF)[113] of IgG-Fc bound to CD16, which is represented in a simplified form in **A** In purple is the D1 and D2 domain of CD16 while the green and red colors illustrate the two respective heavy chains of the IgG molecule and the glycans are illustrated as well. Made using biorender.com.

Fc-γ receptors engage human IgG predominantly through their lower hinge region

In the early 2000s significant advances were made in understanding how antibodies exert their effects[114]. Crystallization studies of antibodies with various Fc-γ receptors revealed that IgG antibodies bind towards the N-terminal domain of the respective receptors in the start of the protein), referred to as immunoglobulin-like domains 1 and 2 (**Figure 7A-B**)[115]. CD64 has an additional third domain unlike the low-affinity CD16 and CD32, and it has been shown to indirectly be involved by stabilizing the interaction of D1-D2 with the Fc of IgGs[115]. The primary engagement occurs between these domains and the lower hinge region at the start of the CH2 domain of IgG antibodies (specifically residues cysteine 228 to proline 238). The interaction is dependent on salt-bridges and hydrogen bond formation predominantly in the lower hinge region for all three Fc-receptors (although inter-receptor differences are prominent) [115].

In addition to structural interactions, glycosylation of IgG antibodies is crucial for enhancing Fc-receptor binding[116]. They stabilize the interaction by filling in the

cavity created between the two CH2 domains. This is theorized to stabilize the mobile lower hinge regions into a confirmation which is preferable for efficient Fc-receptor engagement [116]. Nevertheless, through manipulation of the lower hinge region and other engagement sites with receptors, one can enhance or decrease the activities of these receptors. Interestingly, *Streptococcus pyogenes* utilizes the dependency of lower-hinge interaction with FcR action by directly cleaving the lower hinge of antibodies by an enzyme called IdeS, thereby potentially evading the humoral adaptive response[117,118]. Thus these conserved binding patterns of human IgG to their Fc-receptors can be taken advantage of by pathogens to evade adaptive immune response. This will be discussed further in **Chapter 5**. How then do the binding of FcR to the IgG-opsonized complex lead to antibody effector function? This is an intriguing subject, which we will dive deeper into in the context of antibody dependent phagocytosis in the following section.

Clustering and cross-linking of Fc receptors are essential for phagocytosis

Phagocyte engagement with an invading pathogen during phagocytosis shares similarities with the immunological synapse[119] . For a phagocytic cup to be formed (see **Chapter 1** for details on phagocytosis) there needs to be intracellular signaling which coordinates the actin cytoskeleton to form a pseudopod which surrounds the target. Unlike other receptors, CD32a contains an ITAM motif in its intracellular domain and undergoes phosphorylation upon ligand binding. However, CD16 and CD64 require an adaptor protein, known as Fc-γ subunit to achieve this [119]. The initial phosphorylation is done by the Src Kinase family which leads to an intracellular signaling cascade. These signals lead to the rearrangement of the cytoskeleton leading to the formation of the phagocytic cup and internalization of the phagocyte [119]. It is worth noting that not all Fc-receptors that bind IgGs lead to function, since activation is a multi-step process involving several other players and distinct mechanisms. Furthermore, the presence of CD32b on the effector cell would also influence the amount of cell activation given its role as an inhibitory receptor [119].

What is then required for efficient activation of the Fc-receptors? Similarities exist with the immunological synapse, where clustering of T-cell receptors (TCRs) in the central supramolecular activation cluster (cSMAC) is a strategy to co-localize signals and physically remove phosphatases such as CD45[59] . It has been shown that in the phagocytic synapse, Fc-receptors need to be clustered and phosphorylated for efficient phagocytosis which happens through several interacting mechanisms. One, is that multiple IgG's need to be present closely on an immune complex[120] which enables binding of multiple receptors at the same time, known as cross-linking[121].

Cross-linking has been proposed to be enhanced when Fc-receptors are mobile in the membrane [122], which can be modulated by previous IgG-FcR activation[123]. However, generally the FcRs are much less mobile and fluid in the cell-membrane than the TCRs and cannot concentrate into the same high concentrations as TCRs in the cSMACs[124]. This can be compensated however through integrin activation by complement receptor 3 and 4 (CR3 and CR4). Integrins promote CD45 removal from microclusters[125]. CD45 is a phosphatase that inhibits Fc-receptor signaling through dephosphorylation. Integrins are activated by Fc-γ receptor signaling (inside-out signaling)[126] which subsequently can lead to both pathogen binding (some integrins such as CR3 and CR4 exhibit a promiscuous binding profile to diverse proteins and structures) but also removal of CD45 away from the Fc-receptor microcluster [125]. The former increases the total avidity of the phagocyte-target interaction (similarly as with T-cells and DCs) which is important given that Fc-receptors are contained in sparse numbers in distant microclusters and thus serving as a bridge between them. The extent of the FcR mobility[127] in cell membranes, and its significance for ADCP, is being investigated and it is not fully understood how much mobility they do exhibit. For instance, it has recently been suggested that prior IgG priming on macrophages can increase ADCP in subsequent encounters (after 1 hour) through increased FcR mobility [123].

Several of the above mentioned mechanisms are independent of antibodies. However, the intrinsic nature of the antibody constant domain and its epitope are crucial factors for initial antibody-FcR engagement, cross-linking and function. While we have discussed molecular affinities to FcR and how subclass differences exist, other important aspects warrants attention. Of note, it has been shown that the distance of the epitope to the cell-surface of the phagocyte directly influences the phagocytosis efficiency. Bakalar et al elegantly demonstrated that antigen heights above 10 nanometer from the cell-surface impairs ADCP[128]. A mechanism behind this decrease ADCP is the removal of CD45. CD45 has a large extracellular domain and is sterically removed when the opsonized target is closer to the phagocyte surface similarly as with the immunological synapse between T-cells and DCs (where it is removed to the distal SMAC)[129] . Having discussed in detail about how antibodies activate their receptors, and the importance of clustering and formation of phagocytic synapse for efficient phagocytosis, we can now end the chapter by an overview of other antibody Fab and Fc-mediated functions.

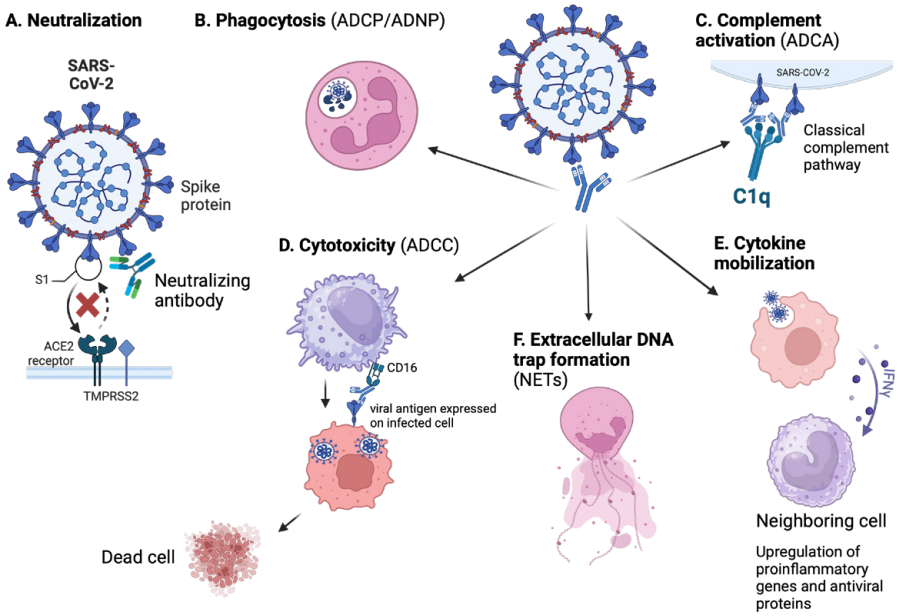

Figure 8. Overview on antibody effector functions. Figure 8 illustrates various Fab and Fc-mediated functions of antibodies**.** In **A** is an example of neutralization of SARS-CoV-2 virus where the spike protein cannot interact with human ACE2-receptor for viral entry due to blocking by a neutralizing antibody. **B** illustrates a diverse set of functions mediated by the Fc domain of the antibody, with examples in a SARS-CoV-2 setting. Image is taken from review Trends In immunology by Izadi and Nordenfelt[130] .

ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY

ADCC can be mediated by macrophages, neutrophils and NK-cells. NK cells are believed to mediate a significant portion of ADCC due to the absence of the inhibitory CD32b Fc receptor and the presence of activating receptors[131] . The ADCC activity in turn is mediated by antibody binding to CD16a present on NK-cells (**Figure 8)**. Several crucial steps must occur for antibody-mediated killing of infected or tumor cells. Firstly, the NK-cell needs to anchor itself to the target cell which has antibodies bound to it. As with the T-cell immunological synapse, the NK-cell needs to form stable interactions with the target cell, which is thought to be mediated by integrins such as LFA-1[132] . Subsequently, the NK-cells will then receive an input of signals through various receptors to perceive if the cell is a friend or a target needed to be eliminated. In the example of viral infections, MHC-1 molecules of infected cells are often downregulated[28] . This has the effect of NK-cells not recognizing the infected host-cell as a friend and proceeds with elimination through lytic content that induce apoptosis. There are other activating receptors

which the NK-cell expresses which can recognize detrimental changes in host cells (such as when a host-cell has become a cancer-cell). Similarly, the NK-cells can recognize opsonized foreign antigens on the surface of infected cells through their CD16a receptor. Subclass is a crucial factor for ADCC, where IgG1 and IgG3 have been shown to be stronger inducers of ADCC than IgG2 and IgG4 through increased affinity for CD16[133]. Similarly, glycosylation of IgGs also strongly influences affinity to CD16 (one prime example is afucosylation of the CH2 domain)[134]. However as discussed before, the interactions with CD16 is primarily thought to be mediated by formation of localized and clustered immune complexes on the target cell. This process leads to efficient clustering and phosphorylation of receptors, analogous to what occurs in the phagocytic synapse and other immune synapses, resulting in cell activation[135]. Through antibody ligation of NK-cells to target-cell viral and bacterial (for intracellular bacteria) can be cleared with the release of cytotoxic granules (containing perforins and granzymes) by the cell.

Complement activation - the classical way

While the subject of ADCP and ADCC has been already covered, we have not mentioned other important downstream functions of antibody binding to antigen. One important function is the activation of the classical complement pathway, which occurs through the binding of C1q to the CH2 domain (**Figure 8)**[97]. IgG4 has a very low capacity of activating this cascade, and IgG2 does so much less potently than IgG1 and IgG3[136]. IgG3 has extensive allotypes, more than 13 known ones, and it could be that some allotypes are more potent inducers of C1q activation than others[137]. While both IgG1 and IgG3 are more pro-inflammatory than IgG2 and IgG4[97], determining which of these two subclasses is the superior activator of this pathway requires further research. Of note, for efficient C1q binding, avidity plays a large role, where many IgG Fcs are needed to activate C1q[138,139]. This is because C1q has approximately 10^{-4} Molar in affinity to IgG Fc, and upon antibody clustering on antigens the avidity is strong enough for binding, where hexamer formation (ratio of six antibodies per C1q) has been shown to be the crucial. Recent evidence has shown that IgG3, due to its extended hinge region, can better initiate hexamer-formation and activation of C1q suggesting that it in fact is better than IgG1 in this regard[140]. Albeit more extensive work is needed.

Several downstream effects occur upon C1q binding to the antibody. The generation of C3a and C5a is important for recruiting leukocytes to the site of infection through chemotaxis, enhancing their pro-inflammatory activity (e.g., increased ROS production and cytokine production by macrophages, and more efficient phagocytosis)[141,142]. Furthermore an important function of the classical complement

pathway is also opsonization. The cascade generates C4b and C3b, which are deposited on pathogens bound by IgGs. These complement proteins, particularly C3b, promote phagocytosis by platelets, neutrophils, and macrophages through complement receptors on these cells. CD35 (CR1) is one high-affinity receptor for these proteins, and is expressed on all peripheral blood cells. It is highly expressed by FDCs and consequently binds the immune complex for antigen display during BCR selection and activation as discussed in **Chapter 1**. Furthermore, CD21 (CR2) is another receptor which promotes phagocytosis, but it only recognizes subproducts of C3b which have been cleaved by Factor I (iC3bC3d/C3dg)[143] . Integrins are also involved in opsonization where the integrins complement receptor 3 (CR3) and 4 (CR4) recognize C3b and promote phagocytosis. CR3 and CR4 are involved in the immunological synapse and leukocyte migration, playing a multifunctional role in the innate and adaptive immune responses.

Finally, the complement cascade culminates in the formation of the membrane attack complex (MAC) with a multitude of complement proteins which create a pore in the lipid membrane of the pathogen leading to termination by lysis [141,142]. Taken together, upon binding of an IgG antibody to a pathogen can lead to a synergistic elimination of the invader by opsonization (through Fc-receptors and complement receptors 1-4) and enhanced pro-inflammatory activity of leukocytes. Thus it is not surprising that the weaker activation or non-existing activation of the classical complement pathway by IgG2 and IgG4 respectively makes these two subclasses less inflammatory. However, aberrant activation of the classical complement pathway and other proinflammatory systems can lead to detrimental effects on the host. This will be highlighted in **Chapter 5-6** when we discuss hyperinflammation in Streptococcal and COVID-19 patients. It is also noteworthy that certain pathogens (specifically *Streptococcus pyogenes*) have evolved virulence proteins capable of counteracting IgG-induced activation of the complement pathway and IgG-independent complement pathway[144], underscoring their significance in eliminating these pathogens.

INTRACELLULAR ANTIBODY DEPENDENT NEUTRALIZATION

One quite recently discovered immune mechanism by antibodies is the intracellular defense against non-enveloped viruses and intracellular bacteria through the intracellular receptor TRIM21 by engagement of antibody Fc (**Figure 8**)[145]. TRIM21 preferentially binds IgG at the CH2-3 interface (with subnanomolar affinity)[146], its binding to but IgA and IgM is less well-documented[147]. Interestingly, TRIM21 ubiquitinates viral virions, targeting them for proteasomal degradation. This process prevents viral replication and aids in clearing infected cells. In addition

to facilitating degradation, TRIM21 also activates signaling pathways that lead to pro-inflammatory responses. TRIM21 induces NF-κB, AP-1 and IRF signaling pathway which leads to production of pro-inflammatory cytokines, upregulation of intracellular and extracellular receptors important for immune defense (such as MHC molecules)[147]. Through virion degradation, TRIM21 exposes viral nucleic acids to intracellular pattern recognition receptors, triggering additional antiviral mechanisms (discussed in **Chapter 1**). The effects by antibodies through TRIM21 have been called intracellular antibody dependent neutralization or degradation, and the excellent review by Rhodes and Isenberg can be read for more information[145].

NETosis

Neutrophils have the unique ability to expel their DNA as chromatin structures called neutrophil extracellular traps (NETs), resembling nets cast by a fisherman (as discussed in **Chapter 1**)[148]. NETs can have antimicrobial effects on the pathogen but also detrimental effects to the host[21,149]. The fate of neutrophils during NETosis varies; while it often results in cell death, some studies suggest that neutrophils may survive for several hours under certain conditions. While NETs have antimicrobial effects, excessive NETosis has been implicated in inflammatory diseases. In COVID-19 patients, excessive NETosis in lungs was linked to severe acute respiratory syndrome and detrimental clinical outcome[150]. Immune complexes formed by IgG and antigens can induce NET formation via CD16b engagement on neutrophils (**Figure 8**). Interestingly, polyclonal IgA has been shown to be a more potent inducer of NETosis than polyclonal IgGs through Fc-alpha receptor I[151]. These processes highlight the multifaceted roles of antibodies in immune defense mechanisms.

Agglutination - facilitates bacterial aggregation for enhanced phagocytosis

Apart from cross-linking Fc-receptors, antibodies can cross-link bacteria into larger clumps through binding of antigens[152]. This is an important process for prevention of adhesion to host-surfaces and colonization. While IgA is primarily responsible for mucosal immunity, circulating IgG and IgM play significant roles during systemic infections. In mucosal areas, IgA is initially present at high concentrations and plays a key role in preventing bacterial colonization [152]. While IgA dominates mucosal immunity, systemic infections recruit circulating IgG and IgM which can disseminate into inflamed mucosal areas and aggregate invading bacterial pathogens and reduce colonization (albeit less efficient than when having prophylactic protection by preexisting IgA). Furthermore, it has been shown that aggregated

bacteria can be more readily phagocytosed by immune cells [153], through complement and Fc-mediated uptake[154] processes primarily driven by IgG and IgM. Beyond facilitating aggregation and phagocytosis antibodies also directly neutralize pathogens by blocking their ability to infect host cells.

NEUTRALIZATION

While we have discussed the Fc-mediated function of antibodies, antigen-binding per se can lead to protective immune functions. These mechanisms include neutralization of toxins, enzymes and inhibiting effects of membrane bound proteins. Neutralization in this context means that the antibody inhibits/nullifies the effects of the protein it binds to[155]. Classically, neutralization has been centered around toxin inhibition, where antibody binding can through various mechanisms such as direct blocking of the toxin and its substrate or inducing a conformational change leading to loss of functional activity. The neutralizing effects of antibodies were first demonstrated by Emil von Behring and colleagues, who used immunized horse serum to treat diphtheria[156]. Thus one of the major important functions of antibodies is to inhibit pathogens virulence factors and protect the host subsequently.

In the context of viral infections however, focus lies on blocking viral entry into the host T-cells by HIV or ACE2-receptor expressing cells by SARS-CoV-2. The COVID-19 pandemic generated extensive research into neutralizing antibodies against SARS-CoV-2, leading to many important discoveries[155]. It has been shown that antibodies can block viral entry and stop the viral replication cycle (and thus lower infectious titers) through several distinct mechanisms[157]. Given this thesis focus on SARS-CoV-2 this pathogen is a suitable example to discuss regarding neutralization. SARS-CoV-2 utilizes the trimeric glycoprotein spike which contains a receptor-binding domain to interact with human ACE2-receptors highly expressed on epithelial cells in the upper and lower respiratory airways[158]. This binding of RBD to ACE2R induces a conformational change in the spike glycoprotein where one part (the S1 subunit) dissociates and the second part (S2 subunit) is inserted into the host-membrane like a spike (hence its name). The insertion of the S2-subunit promotes membrane fusion of the viral virion with the host cell subsequently leading to viral replication. This process can be neutralized by antibodies through several distinct mechanisms. To mention a few, antibodies can bind to the RBD domain which engages the ACE2-receptors and directly block the interaction[159]. Antibodies can also bind to other sites of the spike protein, such as the N-terminal domain, and sterically (using the Fc tail) block the engagement of RBD-hACE2. Anti-spike antibodies can bind the S1/S2 junction, inhibiting S1 subunit dissociation- a step

critical for S2 conformational changes required for membrane fusion. Finally, anti-spike antibodies can block viral entry into the host cells by binding to the S2 domain, and preventing membrane-fusion after the S2 domain has been inserted in the host membrane. These different mechanisms have their pros and cons, where mutational susceptibility, concentration needed for 50% blocking (commonly used metric is IC50) and cross-reactivity (for instance recognizing other viral pathogens such as SARS-CoV-1) are some worth mentioning. The takeaway from these examples is that the host immune response can generate both opsonic antibodies (antibodies which induce ADCC, ADCP etc) but also antibodies which can exert direct inhibition or blocking of the pathogens virulence through several overlapping and distinct mechanisms[159] . Interestingly, antibodies can have both opsonic and neutralizing function, which depends on their epitope specificity and constant domain class. These diverse neutralization strategies highlight how antibodies directly inhibit viral replication while complementing other immune defense mechanisms. In summary, neutralizing antibodies play a crucial role in blocking pathogen virulence factors such as interfering with toxin activity or viral entry mechanisms.

Authors comment on the Chapter

Hopefully, it has now become clear that antibodies exert their function in a highly regulated manner, where the activation of effector cells is balanced by activating and inhibitory signals, governed by molecular affinity for various receptors, as well as avidity effects. Avidity effects are influenced by inherent constraints in the antibody, such as hinge flexibility, and are also affected by antigen density, epitope distance, and the valency of the immune complex. Understanding this, we can design novel therapeutics that leverage these complex biological mechanisms to benefit the patient. To test antibody function, we first need to obtain the genetic sequence to express these antibodies recombinantly in the lab. The next chapter will introduce the antibody discovery process, highlighting the contributions of historic giants such as Cesar Milstein. As we will see, the process was long and complicated, but as a famous physicist once said:

"In the middle of difficulty lies opportunity." – Albert Einstein

CHAPTER III
DISCOVERY OF MONOCLONAL ANTIBODIES

Introduction to the chapter

Having discussed how the process by which antibodies are made *in vivo* and their diverse functions in humans, this chapter delves into harnessing these proteins for therapeutic applications. Discovering a clinically viable monoclonal antibody requires meeting several critical criteria. First, antigen specificity is crucial; the antibody should not exhibit promiscuous or nonspecific binding to other targets or cross-react with human tissues[160]. Moreover, if derived from non-human sources, the antibody must be engineered to minimize immunogenicity. Secondly, the antibody of interest needs to be able to be produced in large quantities efficiently in commonly used cell-lines (such as Chinese hamster ovary cells, CHO-cells)[160]. Finally, the antibody must possess favorable pharmacokinetic (e.g., half-life) and pharmacodynamic (e.g., target engagement) properties within the human body. Several strategies exist to address these challenges, such as through careful selection of antibody discovery technologies and engineering strategies. The issue of immunogenicity can be addressed by isolating reactive B-cell clones from human donors to remove steps of needing to humanize the antibody to avoid immunogenicity to the animal IgG backbone[160]. Alternatively, precise mutations in the IgG Fc region can modulate effector functions, such as reducing inflammatory potential by decreasing affinity for Fc receptors. These examples serve to highlight to you the reader the importance of antibody discovery technique, the screening method for determining what antibody clone is of interest and how engineering of antibodies can modulate monoclonal antibodies to be even more efficacious. This chapter will start by describing a historical achievement, the first instance of how antigen-specific monoclonal antibodies could be widely produced for therapeutic development- *the hybridoma technology*!

HYBRIDOMA ANTIBODY DISCOVERY- ANTIBODIES FOR EVERYONE

The wide-spread use of specific antibodies derived from one clone of B-cell, a so-called monoclonal antibody (mAb), was enabled through the groundbreaking work of pioneers César Milstein and Georges Köhler. In their first study, published in 1975, they developed a protocol in which mice were immunized with an antigen

(sheep red blood cells), and B cells were subsequently harvested from the spleen[161]. They then immortalized the B-cells by fusing them (using polyethylene glycol) with cancerous myeloma cells, allowing this new cell (called hybridoma) to survive and secrete antibodies (the antigen specific BCR of the original B-cell).

To selectively culture hybridomas, the authors used HAT medium (hypoxanthine, aminopterin, thymidine)[161]. Myeloma cells lack the necessary enzyme to survive in HAT medium, while B-cells lack longevity and die in this medium, thus only hybridoma cells can be continuously cultured in this medium. The authors then screened the hybridomas for functional activity through plaque assay (antibody induced complement-mediated hemolysis of sheep red blood cells in agar plates). Specific hybridoma clones with functional activity could then be isolated and produce vast amounts of monoclonal antibodies with known functional specificity. While Milstein and Köhler revolutionized antibody research, other researchers like Brigitte Askonas also contributed significantly by isolating plasma B cells with known antigen specificities[162]. However, the Nobel Prize-winning discovery (1984, shared by Milstein, Köhler, and Niels Kaj Jerne) of the hybridoma technique was truly novel was that it allows production of specific monoclonal antibodies in large quantities in a reliable manner, whereas existing methods produced either nonspecific antibodies or insufficient quantities. The first monoclonal antibody which was approved by the FDA (in 1986) used the hybridoma technique, muromonab-CD3, targeting CD3 on T cells to prevent rejection in kidney transplant recipients[163].

HUMANIZATION OF HYBRIDOMA-GENERATED MURINE MABS

It is worth noting that muromonab-CD3 is of murine nature, that is, both the constant and variable domain are from mouse[163]. This has implications for both immunogenicity (human immune system can react to the murine antibodies as foreign substance and mount an adaptive immune response against it)[160] and pharmacokinetic (murine Fc can interact with human Fc-receptors)[164]. The process of humanization was thus a natural progression in the monoclonal antibody field. The first step was done by Gregory Winters team where they cloned the variable domain of the murine antibody into a plasmid framework containing human constant domains[165]. This hybrid of murine-human antibody was called chimera, and the first FDA approved chimeric antibody was done in 1994 (abciximab).

Today, newer techniques such as antibody discovery through B-cell isolation from human donors (more on this in the next section) removes the need for humanization since both the Fab and Fc are fully human. In the next section we will discuss the B-cell isolation technique employed for discovery of the monoclonal antibodies

used in this thesis in addition to which cell-line used for production and how these antibodies were purified. Finally, we end the chapter with discussing different monoclonal antibody engineering strategies (particularly Fc-engineering) and their current application in the field.

Recombinant monoclonal antibody production from human isolated B-cells

Over time, new technologies emerged that complement or replace the hybridoma technique for antibody discovery. These include immortalization with EBV-virus, phage display and isolation of B-cells using fluorescence-activated cell sorting. These techniques have various advantages and disadvantages (see review by A. Pedrioli & A. Oxenius)[166]. In this thesis, monoclonal antibodies were used that were discovered by isolating antigen-specific human B-cells from convalescent patients (patients who after initial infection donated their blood 6 weeks post-infection) using FACS. For the antibody discovery process against Streptococcal M protein, VDJ sequences were obtained via molecular cloning. For anti-SARS-CoV-2 antibodies against the spike protein, next-generation sequencing was used to acquire the VDJ. These different techniques and how they were employed will be discussed below.

Antibody discovery through antigen-specific B-cell FACS sorting and RT-PCR

To isolate antigen-specific B cells, the method must incorporate the antigen, either through prior immunization or by screening B cells using fluorescently labeled purified antigen or antigen-coupled beads[166]. Wrammert et al. isolated antibody-secreting plasma cells (ASCs) producing high-affinity anti-influenza antibodies 7 days post-vaccination[167]. The authors chose this time frame due to the fact that ACSs peaks in concentration before they migrate to the bone marrow, where isolation from human subjects presents logistical and ethical challenges. The authors then enriched B cells via negative selection using RosetteSep, which cross-links all other blood cell types (except B cells) to erythrocytes, facilitating efficient B-cell isolation [167] . Thereafter, the B-cells in their entirety are analyzed in a flow-cytometer using antibodies against different CD-markers (these anti-CD antibodies are fluorescent) (**Figure 9A-B**). A flow cytometer is a device used to analyze physical characteristics of cells, such as size and granularity (See **Figure 9C**). Using anti-CD antibodies, cells can be classified based on CD phenotype expression (immunophenotyping).

For instance by using CD3 and CD19 markers we can efficiently divide up an unspecified lymphocyte population in a sample tube by CD3+/CD19- (T-cell) or CD3-/CD19+ (B-cell) (**Figure 9C).** The flow-cytometer can do this by aligning cells individually in a microfluidic channel[168] . Each cell will be subjected to being lasered, where several different lasers can be used in the device with different light spectrums. The flow-cytometer contains detectors which allows analysis of fluorescence, size, granularity and other features. In our example with the B and T-cells, fluorescent antibodies against CD19 and CD3 respectively will bind to the respective cells and emit fluorescence when excited by a laser of appropriate wavelength. Based on size and granularity, the cells can be divided into populations, thereafter using the fluorescent markers, the populations can be further classified. This population selection process is known as gating. Flow-cytometers can also have a sorter, which means that when the gate is set for the population of interest (B-cells for instance) these cells can be sorted into single wells one by one allowing for analysis such as VDJ cloning or sequencing.

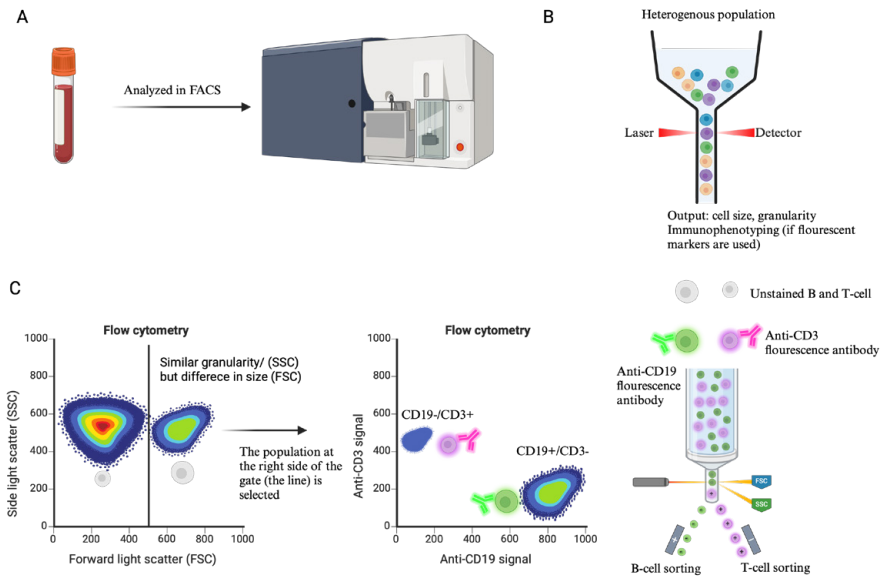

Figure 9. Overview of using FACS for sorting immune cells of interest. Figures **A** and **B** depict the analysis of whole blood in a FACS machine, where lasers illuminate different cell types, represented by various colors. Based on cell granularity, size, and the presence of fluorescently labeled anti-CD markers, a wealth of information can be acquired[168] . This is illustrated in **C** where the bigger cells are selected by drawing a gate separating the two distinct populations (FSC indicating cell size). Using fluorescent anti-CD19 and anti-CD3 markers, cell types can be distinguished based on their emitted fluorescence upon excitation. In the example above, lymphocytes are divided up by CD3 or CD19 expression, into B and T-cells respectively. These can then in turn be sorted/isolated using FACS, allowing for isolation of specific immunological cell types. Made using biorender.com.

As discussed in **Chapter 1,** B-cells express CD19 and CD20, while T-cells express CD3. Thus, by using these initial markers to separate lymphocytes, we can further enrich the B-cell population, even if some T cells or other cell types remain after negative selection with RosetteSep. An initial gate in the flow-cytometer is drawn by using CD3- and CD19+/20+ cells. To study and sort memory B cells, CD27 can be used as a marker, as these cells express low levels of CD38[167]. If ASCs are of interest, their expression of both CD27 and CD38 allows for their separation from CD27+/CD38- memory B cells. To acquire an even better sorting strategy, the authors screened this CD27+/CD38+ population for IgG CSR by using markers for IgD, IgM and IgG respectively (IgD-/IgM-/IgG+). Using a fluorescence-activated cell sorter, the fluorescently tagged B-cell population (labeled via anti-CD markers and BCR constant domain class markers) can be sorted into individual wells or in bulk. In this case, Wrammert et als protocol sorted cells into individual wells. The subsequent step involves obtaining the VDJ sequence of the antibody of interest from these isolated reactive B cells.

After sorting, the B-cells VDJ are cloned into a plasmid vector through multiple PCR steps (**Figure 10A**). Firstly, cDNA of the antibodies is generated from RNA by using a reverse transcriptase and primers specific to a conserved sequence of the antibody gene[167]. A primer is a short segment (15-25 usually) of nucleotides designed to be complementary to sequences one wishes to amplify. After generation of cDNA, further amplification of the VDJ genes is needed. A cocktail of several primers, each specific to different V-gene families (as multiple V-genes exist), is used alongside reverse transcriptase (**Figure 10B)**. This enables the amplification of the B-cell VDJ gene mRNA into cDNA during the first RT-PCR step. To further amplify the V-gene, nested PCR is performed with more specific primers, now that the V-gene family of the amplicon is known (**Figure 10C)**. Nested PCR is a two-step process where one primer pair amplifies the RT-PCR amplicon, and the second primer pair amplifies the resulting product. The amplified VDJ gene segments are then available in large quantities and sent for sequencing to generate specific information. The nested PCR uses primers that generate sequences upstream and downstream of the VDJ genes, which can be used to insert the gene fragments into a plasmid vector using restriction-digestion enzymes. The final step of the protocol results in generation of either one plasmid containing both the heavy and light chain of the antibody (variable and constant domain) or a two-plasmid system where you have the light chain in one plasmid and the heavy chain in the other (**Figure 10D)**. These plasmids are then transfected to a suitable cell-line which can produce our monoclonals. Wammert et al used human embryonic kidney cells (HEK293) to produce these antibodies. Plasmids are transferred to the HEK293 cytosol by transfection using Polyethylenimine (PEI) which gives the plasmids a positive

charge enabling endocytosis leading to transcription in the nucleus and subsequently translation of the antibody (**Figure 10D)**[169] .

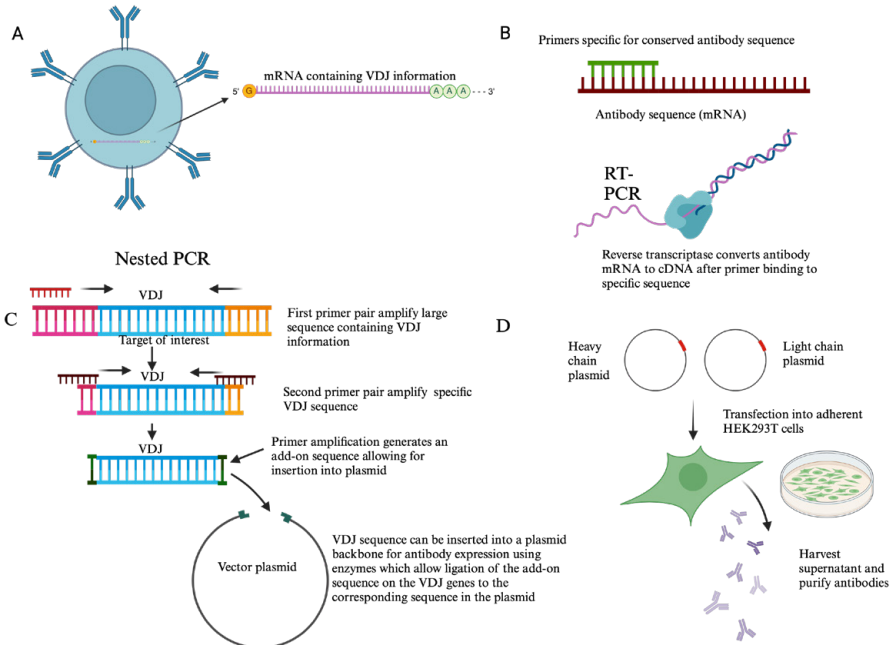

Figure. 10 Antibody production by PCR technology of BCR sequence. Figure **A** depicts a B cell expressing B-cell receptors (BCRs). Within the cell, mRNA molecules encode the variable (and constant) domains of the antibody to be produced during protein translation. Figure B describes the conversion of mRNA into cDNA using reverse transcriptase and primers specific to conserved regions of the antibody variable domain. Figure **C** shows nested PCR using specific primers to amplify variable domain sequences from the cDNA generated in Figure **B**. The final PCR reaction leaves the amplified sequence with a restriction digestion sequence, shown in dark green, which allows insertion into a vector plasmid containing the constant domain gene (and other necessary genetic sequences for successful translation, such as promoter). **D** Shows how the complete heavy chain plasmid and light chain plasmid are added to adherent HEK293T cells which produce the monoclonal antibody. The antibodies are secreted into the supernatant, ready to be purified and collected, using methods such as protein G beads which specifically bind to the Fc region of IgGs.

This method offers several advantages over the traditional hybridoma technique. Firstly, these are human antibodies, and thus there is no need for humanization. Secondly, this method is faster and can be done in 28 days only, while traditionally hybridoma takes longer. Thirdly, this method captures the immune response by natural infection in humans, which can guide vaccine design (that is allow us to understand which antigens and epitopes generate an antibody response). Furthermore the technology allows efficient studying of different B-cell populations

and the response to specific antigens. However, this method can be technically difficult, requires access to human or animal blood, and presents challenges when scaling up, particularly during the transfection and PCR steps. Nevertheless, the technique of FACS sorting reactive B-cells has led to great discoveries, of which one was the discovery of the first human monoclonal antibody against the M protein of *Streptococcus pyogenes* by Bahnan et al (relevant for Paper II of this thesis)[170].

Using the technique established by Wrammert et al and others, FACS sorting is followed by PCR-based VDJ gene isolation and amplification, Bahnan et al isolated reactive B-cells from a patient who had a Streptococcal pharyngitis infection[170]. The aim was to generate antibodies specific to the M-protein of the bacteria, which is a critical virulence factor allowing immune evasion and invasion of the host (amongst other things). The authors produced the antigen bait of interest, the M-protein (of M1-serotype), by recombinantly producing this antigen and fluorescently tagging it. They isolated B-cells as done by Wrammert et al, but the gating strategy differed. Here Bahnan et al, used first a live-dead stain to remove unwanted cell-debris. Then they used a CD19 and CD3 marker to exclude T-cells and include only B-cells. Unlike Wrammert et al, Bahnan et al did not utilize a CD27 nor CD38 marker to further subdivide the B-cell response to the M-protein. Instead the authors assessed the CD19+/CD3- population reactivity to purified M1-protein and the +population which also expressed IgG+ BCRs were isolated by FACS into single wells. The VDJs of these M-protein positive B-cells were then cloned into the two-plasmid vectors allowing for antibody production in HEK293T cells using the PEI transfection method. Interestingly, 10 antibodies were produced, where 3 exhibited nanomolar affinity to the M-protein. Of these, only one was shown to mediate ADCP. This particular antibody, called Ab25, will be discussed in more detail in **Chapter 5** and Paper II of this thesis.

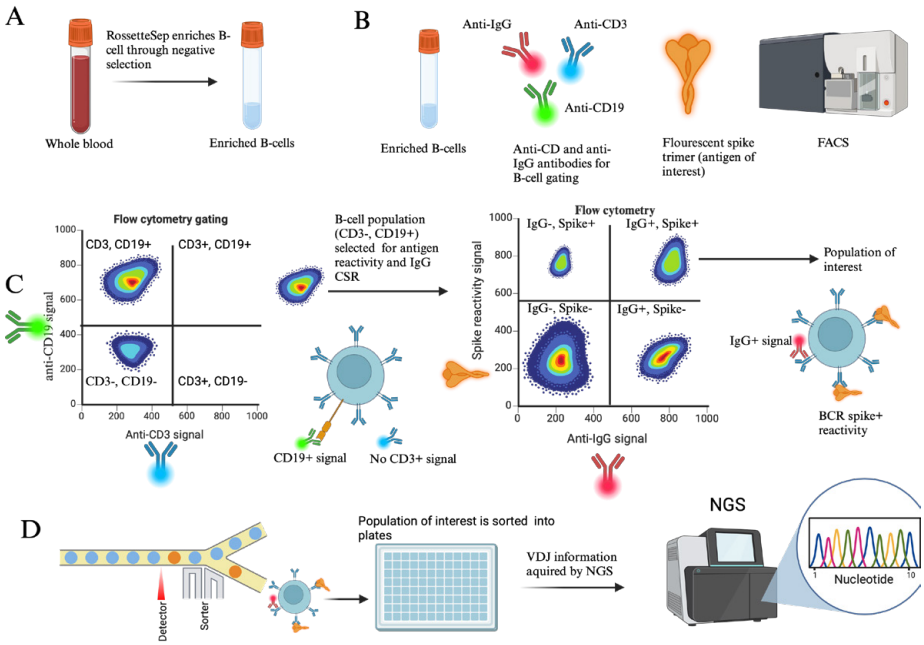

Figure 11. B-cell sorting through FACS antigen-baiting and NGS sequencing. A Whole blood is collected from donors, and B-cells are enriched using a kit called RosetteSep, which cross-links all other cells to red blood cells, leaving the B-cells untouched. Fluorescent antibody markers, each with a different color and specific for IgG, CD3, and CD19, are then added to the cells, along with the spike trimer (the antigen of interest). Class-switched B-cells (IgG+) which are reactive to the spike antigen will be identifiable using these markers, resulting in a CD3-/CD19+/IgG+/Spike+ as seen in **C.** The final gate for this population is set, and the B-cells are sorted in bulk (or individually) into plates, as shown in **D**. The B-cells on the plates are then sent for next-generation sequencing to obtain the VDJ sequence information for both heavy and light chains. The VDJ sequence is then inserted into empty vector plasmids and transfected into appropriate cell lines for antibody expression. Made using biorender.com, the illustration depicts the strategy used for B-cell isolation from Bahnan et al [171] .

ANTIBODY DISCOVERY THROUGH ANTIGEN-SPECIFIC B-CELL FACS SORTING AND NEXT-GENERATION SEQUENCING

As high-throughput sequencing advanced, the antibody discovery pipeline evolved. Next-generation sequencing became a valuable tool for methods such as phage display and B-cell sorting by FACS, providing extensive information on the antibody repertoire post-infection or for mAb development[166] . While the PCR protocol is cheaper in terms of reagents, NGS sequencing of B-cells (via the FACS method) can be more cost-effective when factoring in labor costs. Additionally, the NGS method generates vast amounts of data on BCR sequences, allowing for bioinformatics analysis and modeling of the antibody sequence prior to production.

This is particularly useful for acquiring highly mutated CDR3H and L antibodies, as NGS provides data from bulk sequencing of thousands of cells [166]. Performing thousands of PCRs can be labor-intensive, even if it provides sequence information. During the COVID-19 pandemic, this method of acquiring monoclonal antibodies was widely adopted by various labs. Similarly, the lab of Pontus Nordenfelt generated 10 monoclonal antibodies with nanomolar affinity for the spike protein of SARS-CoV-2.

In this project by Bahnan et al, the authors acquired convalescent blood 6 weeks post hospital discharge from 7 severe COVID-19 patients (defined as needing high-flow oxygen non-invasive oxygen therapy)[171] . This was in March 2020 when the original Europe B1 strain was circulating in Sweden. The patients' B-cells were isolated in the same manner as in the M1-project described above, with one important distinction. In this project, the authors pooled all the isolated and enriched B-cells (post-RosetteSep), and the spike trimer-reactive B-cells (CD19+/CD3-/IgG+) were sorted together, rather than into individual wells (**Figure 11A-D**). Subsequently their VDJ were sequenced by next-generation sequencing (10X genomics). From a total of 7,000 cells, approximately 600 full antibody sequences were generated, including paired heavy and light chains, along with information on the VDJ family germline genes. The authors then performed bioinformatic analysis to select 96 antibodies based on phylogenetic distribution, and used a service from Twist Bioscience to clone the VDJ into an IgG1 plasmid vector for heavy and light chains. Of these 96 antibodies, 10 exhibited strong reactivity to spike protein trimer and one was a potent neutralizer, and all 10 promoted ADCP when tested. These are just two examples of many projects aimed at generating monoclonal antibodies for therapeutic development, studying the adaptive immune response, or for vaccine design.

Author comment on the chapter:
Having mastered the art of antibody discovery using various techniques, along with emerging technologies such as direct discovery by analyzing antibodies in serum at the protein-level or using AI to generate de novo antibody designs, researchers have begun to investigate what makes an antibody effective. By understanding how monoclonal antibodies exert their effects, researchers can modulate these effects through antibody engineering. Advancements in antibody discovery have propelled antibody engineering, which will be discussed in the next chapter. None of this would have been possible without pioneers like Milstein and Köhler, who paved the way years ago.

CHAPTER IV
ANTIBODY ENGINEERING FOR TAILORING THE IMMUNE RESPONSE

Introduction

The field of antibody engineering began with the humanization of monoclonal antibodies (mAbs), where mAbs derived from murine hybridomas were initially generated as human-mouse chimeras (featuring a human Fc backbone and murine variable domain)[165]. Subsequent advancements shifted the focus toward generating either pro-inflammatory immune responses to eliminate tumors[32] or reducing inflammatory responses when only blocking/neutralization was desired. The chosen antibody engineering strategy depends on the intended interactions of the mAb. For instance, if increased antibody-dependent cellular cytotoxicity (ADCC) is desired for an anti-tumor mAb, then increasing affinity for CD16a (expressed on NK cells) is a suitable approach [32]. Conversely, to avoid inflammatory reactions and solely block the activity of inhibitory molecules on tumors (such as with anti-PD-1 antibodies), reducing affinity for FcRs and C1q is a viable strategy (example)[172] . Along these lines, this chapter is divided into three subchapters illustrating creative approaches to generate promising therapeutic mAbs.

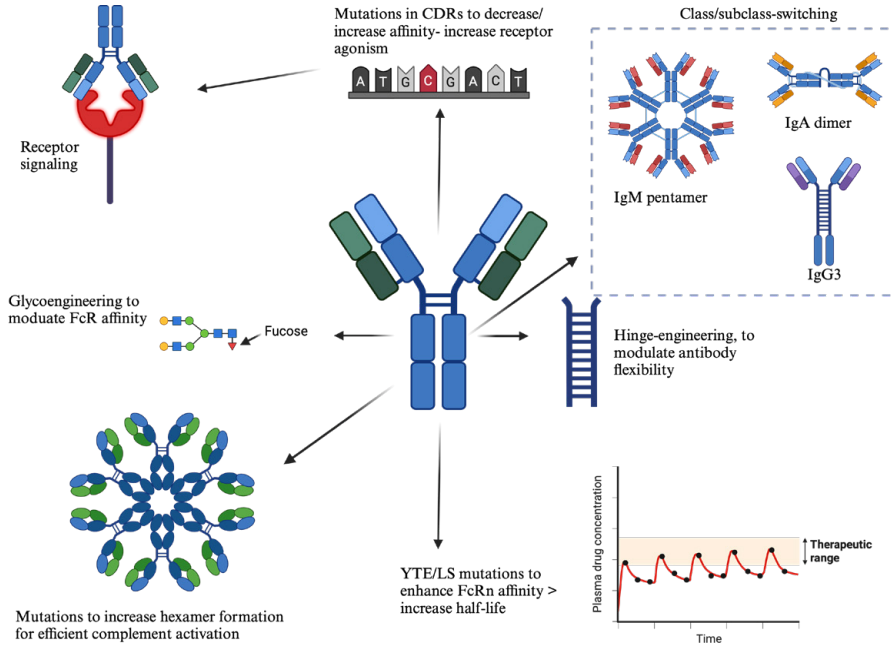

Figure 12. Antibody engineering strategies. The figure illustrates the diverse antibody engineering strategies that can be considered. From top left in clock-wise manner: decreasing affinity can increase antibody agonism when targeting activating receptors such as CD40. Other strategies revolve around changing class for enhancing avidity, such as into IgM pentamers or IgA dimers for enhanced neutralization. Hinge-engineering can be employed to increase antibody flexibility. Point mutations can be induced to change antibody affinity to various receptors, such as the neonatal FcR which influences antibody half-life *in vivo*. Point mutations can be inserted to also increase antibody hexamer formation for efficient C1q activation (dark-blue in the middle). Removal or addition of glycans in the glycan tree at the CH2 domain can abrogate, decrease or enhance FcR affinity profiles.

VARIABLE DOMAIN MODIFICATIONS FOR INCREASED ANTIBODY FUNCTION

As discussed in **Chapter 2**, Fc-clustering is important for efficient activation of effector cells, leading to ADCC and antibody-dependent cellular phagocytosis (ADCP). To achieve this *in vitro* and, to some extent, *in vivo*, strategies have been employed to increase the proximity of IgGs to each other once bound to the target[112] . This can be achieved by designing bi- or multi-specific antibodies, which contain two or more variable domains specific to different epitopes on the same or different antigens displayed, for instance, by a cancer cell[173]. Several different techniques exist to generate bi- or multi-specific antibodies (please see review [173]), an approach gaining popularity in cancer therapeutics. Bi- and multi-specific

antibodies also gained prominence in the field of infectious diseases during the COVID-19 pandemic, where *in vitro* neutralization of SARS-CoV-2 was more potently demonstrated when mAbs could bind to non-overlapping receptor-binding domain (RBD) domains[174]. Similar results have been shown with HIV in macaque animal models[175]. Regarding Fc-dependent functions, designing multi-epitope specific mAbs has been shown to increase complement activation and ADCC/ADCP through increased Fc-receptor clustering (since more antibodies will be present on the target surface without competing to the same extent as the same amount of mono-specific mAbs)[173]. Other innovative uses of bi-specific monoclonal antibodies are the so-called T-cell engagers. By having specificity to CD3 and the other fab specificity directed towards the tumor cell (or target cell), the bispecific mAb can increase the killing of the targeted cell of interest by bridging CD8+ T-cells with the target[176]. In these mAbs an Fc-silent mAb is desirable, to not induce killing of the T-cells themselves through ADCC[177] . These T-cell engagers have been developed for Acute lymphatic leukemia (ALL), where the Bispecific mAb binds to CD3 and CD19 [176]. Given the importance of NK-cells for *in vivo* ADCC, NK-cell engagers are being developed in a similar fashion as the T-cell engagers (but instead of CD3 the specificity to NK-cells is acquired by targeting CD16)[178].

There are other Fab-based engineering strategies which deserve attention. A less intuitive approach involves reducing affinity for the antigen target via mutations in the VDJ-region/CDRs, as demonstrated by Yu et al[179]. The authors decreased affinity of agonistic antibodies targeting CD40 (the binding to CD40 by anti-CD40 mAbs induces intracellular signaling). They observed that antibodies with lower KDs, due to faster dissociation from the ligand, increased CD40 receptor clustering, allowing for stronger intracellular activation and increased survival in murine models challenged with murine lymphoma cells (**Figure 12**). However, in the same study, this approach led to worse ADCC and ADCP by another set of monoclonal antibodies targeting a tumor necrosis factor receptor (4-1BB). Thus, careful selection of function needs to be considered before engaging this strategy, where low-affinity antibodies might promote agonistic receptor activation of the ligand but induce weaker Fc-functions by FcRs. The above examples illustrate the creativity of engineering the variable domains to target multiple epitopes to increase Fc-clustering or by increasing neutralization. Now we will discuss some engineering strategies targeting the Fc specifically.

ANTIBODY FC-ENGINEERING STRATEGIES TO MODULATE ENGAGEMENT WITH FCRS

GLYCOENGINEERING

One popular strategy to increase antibody Fc-effector function is the alteration of the glycan composition at the CH2 domain (which interacts with the Fc-receptors as discussed in **Chapter 2**). In 2004, two crucial studies significantly advanced this subfield. Firstly, the study by Okazaki et al[180] showed that the removal of fucose from an IgG1 mAb enhanced affinity for CD16a more than 20-30 fold, which was attributed to increased binding to the ligand rather than decreased dissociation. This increased CD16a affinity correlated with increased ADCC function, showing that glycan modification (glycoengineering of the Fc) is a viable approach to enhance ADCC of mAbs (**Figure 12**). Similarly, the study by Niwa et al[181] in the same year showed that afucosylated rituximAb (an anti-CD20 mAb which depletes CD20+ B-cells) elicited greater ADCC (10-100 fold) by PMBCs from 20 donors compared to the wild-type rituximAb which contained more fucose. The increased effect by the PBMCs was attributed to N-cell activity, which is the main mediator of ADCC *in vivo* as discussed in **Chapter 1**.

Concerning ADCP, macrophage phagocytosis can be enhanced by afucosylated IgG mAbs due to increased CD16a affinity. Unsurprisingly, given that CD16b shares 97% homology with CD16a, afucosylation also increases affinity to this receptor[182,183]. CD16b is a crucial receptor for ADCP by neutrophils. Thus, removal of focuse can both increase ADCC by NK cells and increase phagocytosis by macrophages and neutrophils through enhanced CD16a and CD16b affinity, respectively. A promising example of the use of afocusylation is illustrated by Mogamulizumab (KW-0761), an antibody which targets Chemokine receptor 4 (CCR4) expressed by T-cells, used for T-cell lymphomas[184]. Another interesting case is that of avelumab, an anti- PD-L1 mAb, where the authors showed that in a Fc-receptor humanized murine model, afucosylated PD-L1 mAb reduced tumor load compared to wild-type and Fc-null versions[185]. Thus, even though the mechanism of action was by blocking inhibitors of the immune response, that is by being a so-called checkpoint inhibitor, enhancing Fc-function by glycoengineering was shown to be beneficial for the overall anti-tumour response. Removal of the glycans completely can abrogate binding to FcRs as shown by Walker and Colleagues in the late 1980s[186], which is suitable for mAbs where pro-inflammatory effects are not suitable (for instance in rheumatoid disease). Mutation of the N297 residue can completely abrogate the glycosylation of the CH2 domain, resulting in

loss of FcR and C1q binding[187]. This strategy has garnered attention in mAb clinical trials, but it remains to be determined the feasibility and success of such a strategy.

Many bacteria produce enzymes which can remove the glycans on antibodies. One notable example (relevant for this thesis) is the enzyme EndoS which is produced by *Streptococcus pyogenes*[188]. The existence of enzymes which can remove Fc-glycans of mAbs to dampen inflammatory response has not been used. However, a clinical indication is their use to dampen the inflammatory activity of patients' own polyclonal antibody response in autoimmune disease. Recently a novel endoglycosidase was discovered which is produced by *Corynebacterium*[189], called CU43, and it was shown by Sastre and colleagues to have promising preclinical effect on several types of IgG-mediated pathologies such as autoimmune hemolysis and antibody-dependent enhancement of dengue infection (in murine models). Apart from glycoengineering, most engineering efforts revolve around inserting mutations in the Fc constant domain, manipulating the hinge domain or altering the constant domain entirely (class-switching artificially). These engineering strategies will be discussed in the next section.

CLASS-SWITCHING

Typically after a hybridoma mAb has been generated it is commonly inserted into a murine IgG backbone or a human IgG1. With improvements in cloning techniques using restriction-digestion enzymes, antibody plasmids which contain the heavy chain constant domain could easily be altered to express other classes and subclasses (**Figure 12**)[190]. In the infectious disease field, subclass-switching from IgG1 to IgG3 has been done by several different groups for anti-HIV antibodies[191,192]. They showed that ADCC, ADCP and complement deposition could be potentiated (in human *in vitro* and *ex vivo* settings) by altering subclass from IgG1 to IgG3. However, improvements *in vivo* were not demonstrated, which can be due to difficulties in comparing cross-species (human antibodies in a murine immune system for instance) [191,192]. Interestingly, Stapelton et al[105] altered the subclass of IgG1 to IgG3 for an anti-pneumococcal mAb, which showed greater efficiency, by lowering bacterial load in blood, *in vivo* compared to IgG1 (in a murine model of disseminated pneumonia) when the half-life of the IgG3 mAb was increased to that of IgG1. However, a half-life enhanced IgG3 construct was shown to be inferior to the original IgG1 mAb when used in a murine melanoma *in vivo* model[193] (it was also inferior in promoting ADCP), highlighting that this approach is perhaps more context dependent. IgG1 comparisons have also been made with that of IgM where promising results have been observed in animal models in the context of *E.coli* and Group B *Streptococcus* bacteria[194]. In the

study by Raff et al, the authors demonstrated that IgM potently protected rats against both pathogens when the IgG1 versions were not [194]. Another study which demonstrates the importance of not preemptively ruling out less-inflammatory mAbs is a study on *Neisseria meningitidis*[195]. Here the authors demonstrated that IgG1 mAbs against an antigen (porA) which is displayed on the surface of the bacteria at high density where more bactericidal than the IgG3 versions, and the opposite was observed for an antigen (factor H binding protein) which are more sparsely present [195]. This study by Giuntini et al highlights the importance of studying each antigen-antibody context independently of previous findings and not extrapolating too much from other contexts.

Although switching from IgG1 to IgG3 constant domain has been a popular approach in the context of Fc-mediated function, altering the constant domain to IgA and IgM has also been widely employed when enhancing neutralization has been the aim. This is particularly true in the context of SARS-CoV-2, where dimeric IgA (two identical IgA mAbs linked together with a linker called the J-chain) and pentameric IgM (five IgM molecules linked together by the J-chain) where much more potent in neutralizing SARS-CoV-2 virus compared to IgG1 versions[196,197]. The increased neutralization potency was in the order of 10-100 fold in these studies, and in the case of IgA dimers, where shown to be protective in murine models of authentic infection (however IgG1 was not compared, regrettably, in this study)[196] . Interestingly the enhanced neutralization potency was also observed by other research groups when engineering SARS-CoV-2 mAbs from IgG1 to IgG3, with a 50-fold increase in neutralization[198]. Even more surprisingly, despite that the IgG1 mAbs lost neutralization function and binding against Omicron mutated variants, changing subclass to IgG3 restored both binding and neutralization function[104] . Similar results were observed with Dimeric IgA against these mutated variants[199] . These studies not only highlight creative strategies to increase neutralization function, they also shed light into how the constant domain influences antibody binding (challenging dogma), as discussed in the opening section of **Chapter 2.**

AMINO ACID MUTATIONS TO MODULATE FCR AFFINITY AND FC-FUNCTION

Given the importance of the amino acids in the lower hinge for binding to FcR, a natural step was to alter the residues engaging the FcRs to modulate affinity[200]. There are countless mutations at various residues which have been studied, and the combination of these leave open many different possible affinity profiles. One of the most used combinations of mutations in preclinical research are the "GASDALIE" mutations: G239A/S332D/A330L/I332E (**Figure 12**)[201]. These

mutations on an IgG1 backbone have been shown to increase the affinity to CD16a and CD32a 10-20 fold, with estimates varying between different studies and mAbs[202] . For the inhibitory CD32b, GASDALIE mutations have been shown to also increase the affinity to this receptor up to 2-fold. In a well-designed study by Bournazos et al, the authors engineered a set of broadly neutralizing anti-HIV mAbs to have the GASDALIE mutations[203] . This engineered variant significantly reduced viral load in mice which were infected with the virus, highlighting the utility of this approach. These findings inspired the use of these mutations later during the COVID-19 pandemic.

In vivo work with SARS-CoV-2 has displayed the utility of using mutations to both study the importance of Fc-function in *in vivo* protection, but also how to enhance protective mAb function by Fc-engineering. There are different combinations that can be used of the G239A/S332D/A330L/I332E, one which has garnered attention is the G239A/A330L/I332E combination, named GAALIE[204] . Using GAALIE mutations the increased affinity to CD32b observed in the GASDALIE variant can be reduced compared to WT IgG1, while maintaining an increase to CD16a/b and CD32. In a well-cited study by Yami et al[157], the authors showed that, when a FDA-approved cocktail of neutralizing mAbs (REGN-cocktail) were given in a therapeutic infection model (infection first, antibody treatment 1 day after), the unaltered WT mAb (the approved version) did not protect mice (mice humanized to express human Fc-receptors) compared to negative control PBS. They then inserted mutations into the IgG1 backbone and observed the change in affinity profile to the Fc-gamma receptors. The REGN GAALIE variants displayed a 9-fold enhanced affinity to CD16a/b compared to WT IgG1, 3.6-fold towards CD32a and a decreased affinity to the inhibitory CD32b (0.3-fold compared to IgG1) and slightly lower to CD64 (0.8-fold). The GA variant had much lower affinity to CD64 (10% of WT) while having 6-fold enhancement to CD32a (with a modest 1.2 and 1.3 fold increase for CD32b and CD16a/b respectively). The ALIE variant, in turn, had enhanced CD64 binding (8-fold), 12-fold increase to CD16a/b and 20% lower to that of CD32a and 20% higher to CD32b. Interestingly, when the GA (G239A) and the ALIE (A330L/I332E) mutant REGN were used in the therapeutic *in vivo* model experiment, they offered slightly greater protection (around 75% survival) than the WT REGN and negative control (40-45% survival) but inferior to the GAALIE variant (100% survival). In a similar study by the same research group, Bournazos et al engineered a GAALIE mutant of an anti-influenza mAb was shown to be fully protective in the FcR-humanized mice challenge model, while the ALIE variant was only slightly more protective than WT IgG1[205]. Interestingly, with this pathogen, the GA mutant mAb was comparable to the GAALIE variant. These results suggested that careful balance needs to be taken into consideration when

balancing the different FcRs, where in the case a reduced affinity to CD32b in combination with more moderate increases to the activating receptors provided best therapeutic outcome. These results also showed that non-neutralizing Fc-function is important for protection against SARS-CoV-2 in mice, and by extrapolating, potentially in humans.

While in the context of SARS-CoV-2, increasing affinity to activating FcRs has been shown to have promising effects in animal models, there are other cases when decreasing or abolishing Fc-function completely is desirable. These instances occur when the purpose of the therapy is to reduce inflammation, such as by targeting inflammatory cytokines in rheumatoid disease. Numerus antibodies have entered clinical trials that express mutations which diminish affinity to human Fc-gamma receptors[206]. Of these mutations, the "LALA" mutations, L234A/L235A, are one variant[207]. Interestingly, in the study by Yamin et al, the authors introduce a set of mutations to create a "Fc-null" REGN variant (abolished FcR affinity)[157]. The mutations are G236R/L328R, named GRLR, and the REGN GRLR mutant was shown to be inferior to the REGN WT, further shedding light on the importance of Fc-function in a SARS-CoV-2 setting. For more information on mutations to modulate FcR affinity please see the excellent review by Saunders[207].

HINGE-ENGINEERING TO MODULATE ANTIBODY FUNCTION

While the initial studies on IgG3 revealed enhanced function, deep mechanistic studies were not pursued[208]. However it was postulated that the extended hinge region allows greater flexibility, enhancing Fc-FcR clustering as discussed in **Chapter 2**. Considering this rationale, studies were undertaken to investigate the effects of hinge-region modifications on antibody Fc-function. One of the first studies on this subject was done by Giuntini et al.[195], which we discussed above. The authors noted that shortening the IgG3 hinge region from 62 amino acids to 17 and 15 amino acids, respectively, significantly enhanced bacterial killing with this truncated version of IgG3 (believed to be due to enhanced complement activation) compared to the WT IgG3. These results were consistent with the results in the same study which compared IgG1 and IgG3, where the longer IgG3 exhibited lower bactericidal killing than the IgG1 version (15 aa). Contrary findings were highlighted by Chu et al in the context of broadly neutralizing anti-HIV mAbs[209]. Here, the authors showed that hinge-length directly correlated with increased ADCP for these mAbs, while ADCC and complement deposition were not affected by hinge length. In another study, hinge length negatively correlated with increased ADCC by IgG3 mAbs[133]. These three studies highlight that different Fc-functions are possibly differently modulated by

hinge-length, where ADCP seems to positively be correlated with hinge length for IgG1 and IgG3, while the opposite seems to be true for ADCC and complement activation does not seem to be influenced based on these three studies. On another note, Trim21-mediated killing of intracellular adenovirus has been shown to be enhanced once IgG3 hinge length modifications where implemented (*in vitro* study)[210]. It is worth noting that none of these studies investigated if these findings were transferable to an *in vivo* model, such as a humanized Fc-R murine model as with the GASDALIE studies mentioned above.

Interestingly, modifications to antibody hinge length have been shown to enhance Fab-mediated neutralization of viral pathogens, particularly in the context of HIV[191,192]. Two separate studies demonstrated that increasing the hinge length from IgG1's 15 amino acid hinge to the longer IgG3 hinge correlated with increased neutralization potency for several monoclonal antibodies (mAbs) in pseudovirus assays. One study focused, by Richardson et al, on mAbs targeting the variable loop domain 2 (V2) of the gp120 antigen[192], while another by Moyo-Gwete et al [191] examined mAbs targeting the V4 region of gp120, both part of the HIV envelope protein responsible for binding to CD4 on T-cells. The authors speculated that the greater flexibility in the Fab-Fab angle provided by the IgG3 hinge, as observed by Roux et al.[103], was responsible for this enhanced neutralization effect. However, these findings contrast with another study by Chu et al.[209], which did not observe a hinge-length dependent effect on HIV pseudovirus neutralization when focusing on mAbs targeting the V3 loop of gp120. These studies, taken together, highlight an important point: the epitope of the target antigen significantly influences the effects of hinge-engineering on neutralization. The studies showing enhanced neutralization focused on broadly neutralizing antibodies against specific regions of the envelope protein (V2 and V4 antigens), while Chu et al.'s study targeted a different region (V3 loop of gp120). These findings underscore the complexity of antibody engineering and the importance of considering epitope specificity when designing therapeutic antibodies, as the impact of hinge-length modifications on neutralization potency appears to be highly dependent on the specific antigen-antibody interaction being studied.

A recent study by Schriek et al compared different engineering strategies on broadly neutralizing HIV mAbs, where GASDALIE mutations and IgG3 hinge-modification and afucosylation was compared for various assays including, FcR binding, ADCP and ADCC[211] . Interestingly, they observed that IgG3 hinge modification potently enhanced ADCP compared to WT IgG1 (while afucosylation and GASDALIE mutations did not). This was attributed to a 1.1-2-fold enhanced binding to CD32a compared to WT IgG1. In this study, ADCC did not increase when IgG3 subclass-

switching or hinge-engineering was done. Interestingly similar binding profiles were observed to CD16a with 1.2 to 2.3-fold increase, which did not translate to an increase in ADCC. Instead, GASDALIE mutations and afucosylation, and both together, was shown to be a robust strategy to kill HIV-infected cells by ADCC. It is worth considering which cell-lines are employed and which receptors these express, for instance the THP1-cell line (used by many to measure ADCP and Shriek et al) express low levels of CD32b which influences the interpretation of the results (for instance when assessing GASDALIE vs GAALIE mutations). Thus these studies further highlight the context-dependent effect on these engineering modifications and the importance of careful characterization using several assays to determine which should be employed in the therapeutic candidate.

ANTIBODY ENGINEERING FOR MODULATION OF THE COMPLEMENT CASCADE

As discussed in **Chapter 2**, the classical complement pathway is initiated by C1q binding to the IgG-Fc domain. This interaction is of low affinity, and successful activation relies on avidity interactions where multiple Fc regions on the opsonized pathogen engage C1q simultaneously, a process called oligomerization[139]. It has been established that six IgG antibodies are typically needed to efficiently activate C1q, a phenomenon referred to as hexamer formation (ordered ring-like structures deposited on the antigen)[138]. Many complement-engineering strategies revolve around increasing the IgG's ability to oligomerize to achieve this effect (**Figure 12**). Notably, some FcR-enhancing mutations, such as GASDALIE, can impair the ability to activate C1q[157].

To promote hexamer formation, several mutations can be introduced. One notable combination is the "RGY" mutations (E345R, E430G, and S440Y)[212]. These RGY mutations enhance hexamer formation by increasing binding between the CH3 domains of adjacent IgG molecules, enabling the formation of the ring-like structure necessary for efficient C1q activation. This approach, named Hexabody technology, has been widely adopted, with several mAbs in clinical trials incorporating this strategy to enhance complement activation[213]. While the RGY approach enhances the avidity of the interaction, other mutations can increase affinity to C1q. These have been shown to result in a 7-fold increase in complement-dependent cytotoxicity (CDC) for engineered Rituximab (anti-CD20 mAb). However, the C1q-affinity mutations also decreased antibody-dependent cell-mediated cytotoxicity (ADCC) for the same mAb, necessitating the introduction of additional mutations to compensate for this effect [213]. Other strategies involve class and subclass-

switching. For instance, IgM, which naturally forms pentamers, provides an inherent avidity-based strategy for efficient complement activation[214].

ANTIBODY ENGINEERING FOR FAVORABLE PHARMACOKINETIC TRAITS

Apart from engineering strategies to increase antibody function *in vivo*, pharmacodynamic traits, and other considerations such as half-life, aggregation tendency and susceptibility to proteolysis must be made[215] . These stability issues are important during large-scale production where antibody purification steps involve exposure to low pH, a factor which influences antibody aggregation, during protein G or A purification from supernatant. Similarly thermal stability is also a crucial factor to consider, which can predict tendency to aggregate *in vivo* and once formulated in higher concentrations[216]. This can be especially important to consider when employing IgG3 as a backbone, given its tendency to form aggregates during large scale production. Saito and colleagues[217] addressed this issue by inserting mutations in the CH3 domain, N392K and M397V, of the backbone, the IgG3 Fc was comparably stable as the commonly used IgG1 backbone.

In the context of half-life, common mutations that increase half-life, by increasing affinity to neonatal fc-receptor (FcRn), are the YTE mutations[218] or the LS[219] (M428L/N434S) mutations[220] (**Figure 12**). Both of these two mutations reduce affinity to human Fc-gamma receptors, but increase affinity for the neonatal Fc receptor. The increase in half-life differs depending on the mAb used, with reports ranging from 2- to 11-fold[221] . For the anti-RSV mAb Clesrovimab, which contains the YTE mutations, the reported half-life in infants was 44 days in a phase 1b/2a trial[222], and around 80 days for adults[223]. For context, the estimated half-life of wild-type IgG1 antibodies in the human body is around 21 days[97]. Interestingly, several FDA-approved monoclonal antibodies for SARS-CoV-2 included either the YTE or LS mutations to increase half-life. For VIR-7832, the reduced CD16a affinity caused by LS mutations was compensated by GAALIE mutations, which enhanced ADCC against infected cells[224]. Thus, engineering strategies can be combined in various ways but this meticulous testing and careful analysis and clear intention on what the treatment is intended to do.

Author comment on the chapter:

Extensive research conducted in the 21st century has generated vast knowledge on how we can modulate antibody function in vitro and, by extension, in vivo. Different engineering strategies allow for the development of custom-tailored products. However, how can we determine which attributes of a mAb are desirable, and how can we extrapolate this to achieve successful outcomes in clinical trials? What is important is studying the disease of interest in a clinical setting, understanding the host-pathogen dynamics to identify the key virulence factors. By combining this with epidemiological studies, such as identifying virulent strains or those more likely to cause disease, we can aim to create mAbs targeting recurring problems, such as the spike protein of SARS-CoV-2. Next, it is crucial to understand what defines protective immunity, such as in the context of anti-spike antibodies, and how we can custom-tailor an anti-spike mAb to achieve the desired effect. These topics will be discussed in the following chapters, focusing on Streptococcus pyogenes and SARS-CoV-2, respectively.

As Sun Tzu wisely said, 'Know thy enemy and know yourself; in a hundred battles, you will never be defeated.

CHAPTER V
STREPTOCOCCUS PYOGENES- A BACTERIA ADAPTED WITH COUNTERMEASURES AGAINST OUR IMMUNE DEFENSE

Introduction to the chapter

Streptococcus pyogenes, also known as Group A streptococcus (GAS), is a human-specific bacterial pathogen. The term Group A is derived from pioneering work by Rebecka Lancefield, where she classified streptococci based on the carbohydrate composition on the bacterial surface and can be used as an antigen test to detect this pathogen[225]. GAS is classified as a gram-positive bacteria, meaning that it has a thick peptidoglycan wall which is stained by a dye called (gram-staining, **Figure 13A**). It also grows in chains, and the shape of the individual bacterium is spherical (**Figure 13A**). Due to production of toxins, it completely hemolysis whole blood (beta-hemolytic) when plated on blood-agar plates. It is responsible for an estimated 750 million infections and over 500,000 deaths annually worldwide[226]. While many infections are mild, *S. pyogenes* is also responsible for severe invasive diseases like necrotizing soft tissue infections (NSTI)[227]. NSTI caused by *S. pyogenes* is a rapidly progressing infection characterized, as the name suggests, by necrosis of underlying tissue due to expression of toxins and uncontrolled bacterial growth. While most infections are non-severe and treatable with antibiotics like penicillin, severe cases can have mortality rates as high as 20-41.4% even with appropriate antibiotic treatment. The clinical spectrum of S. pyogenes infections ranges from common, self-limiting conditions like pharyngitis[228] and impetigo to life-threatening invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome [227]. Rheumatic heart disease, a post-infectious sequel, causes the greatest burden among S. pyogenes-associated illnesses, accounting for 233,000 deaths annually [226].

There is an urgent need for new therapeutics to address acute severe S. pyogenes infections. Challenges in treating severe *S. pyogenes* infections, particularly NSTI, include:

1. Upregulation of virulence factors: S. pyogenes possesses numerous virulence factors that contribute to its pathogenicity[229,230].
2. Serotype diversity: Multiple serotypes exist, complicating treatment approaches.

3. Narrow treatment window: The rapid progression of severe infections limits the time for effective intervention[231].

Recent research has focused on targeting specific virulence factors, such as the M protein, which plays a crucial role in the pathogen's ability to evade host immune responses[232,233]. The development of protective monoclonal antibodies against the M protein represents a promising avenue for future therapeutics, which we will discuss at the end of this chapter, which also involves the present investigation (Paper II).

GAS INFECTIONS IN A CLINICAL SETTING

Studies suggest that approximately 3% and 8% of adults and children, respectively, are asymptomatic carriers of GAS in the pharynx[234] . Other studies suggest it can be up to 20% for children[235]. Thus, GAS can be spread through human contact by healthy individuals without symptoms. From a clinical perspective, GAS is an interesting pathogen because it causes both self-limiting pharyngitis ("strep-throat"), which rarely requires antibiotics, and invasive skin disease with a mortality rate over 20% and a high risk of amputation (around 18%). Antibiotics for strep-throat are usually administered to reduce the risk of complications such as acute glomerulonephritis and rheumatic heart valve disease (RHD), in addition to lower contagiousness[236]. The impact of RHD should not be underestimated, given that approximately 233.000 lives are lost yearly due to this condition[226]. Although invasive GAS disease has a very high mortality and is a major risk of amputation, the incidence rate is low, around 2/100.000 people per year in the US, and globally around 33 million cases[230]. It is not understood why some patients are asymptomatic, while others develop disease.

However, a recent study, by Movert et al[237], shed some interesting light onto this subject. The authors looked at clinical outcomes of patients who had severe cases of invasive skin disease, compared to the more superficial ones, and observed that there was a clear genetic factor in the detrimental cases. Firstly, type 1 interferon response has been linked to immune defense against Streptococcal infection (in mice)[238]. The authors observed that the virulence of the GAS isolates were linked to increased activation of NADase, an enzyme which deprives the host-cell of ATP leading to cell-death adding necrosis and promoting excessive inflammation. Secondly, the authors noted that, of these patients who had a detrimental outcome, they had a distinct allele of a gene encoding an important intracellular PRR. This allelic variant was shown experimentally to be a less potent inducer of the protective type 1 interferon response. Interestingly, another virulence factor was upregulated

in the invasive bacterial isolates, namely the toxin streptolysin O. This protein creates pores in human cells, leading to lysis, and interestingly, allows NADase entry into the host-cells[239]. Furthermore, adhesion of bacteria to the host-cells is mediated by the critical virulence factor the M protein[240] which is required for SLO and NADase to be close to the target cell. Hence Movert and colleagues elegantly highlighted the complexity of host-pathogen interactions, the synergistic activity of virulence factors and taken together shed light into why certain individuals are perhaps more susceptible and predisposed for severe infection[237]. Similarly, the example study strongly demonstrates the synergy of the critical GAS virulence factors for invasive infection and induction of hyperinflammation. This is an intriguing subject, which Victor Nizet and Helena Bergsten wrote an excellent review about[230] . Vaccine research emphasizes on prevention of infection or reduction of severe cases through immunity against virulence factors, passive monoclonal antibody therapy would be more suitable for therapeutic indication once severe infection has occurred. Thus, in the next section, we will briefly discuss the clinical features of NSTI and then focus on important virulence factors for this clinical context and finally discuss recent findings on new therapeutic avenues against this pathogen.

Author note: *It is worth noting that GAS can cause different clinical manifestations of severe infection (such as pneumonia), given paper II focus on NSTI (or using an NSTI-like animal model for GAS virulence) caused by GAS the main focus of this Cappa is on this clinical condition.*

NECROTIZING SOFT-TISSUE INFECTION- FROM A CLINICAL POINT OF VIEW

Necrotizing soft-tissue infections (NSTI) can be caused by polymicrobial infections involving gram-negative bacteria or monomicrobial infections involving gram-positive bacteria. In the former case, patients are typically immunocompromised and the area of infection is usually involving the anogenital-area and head-neck area[241] . In contrast, NSTI caused by GAS typically involves the extremities and thorax-abdomen, often in patients with few comorbidities who are immunocompetent[241,242] . While *Streptococcus pyogenes* can cause superficial infections such as cellulitis and erysipelas, which involve the subcutaneous fat, epidermis, and dermis, NSTI involves deeper infection in the fascia and underlying muscle. The clinical picture can be complex, but common "*red flags*" are:

1. Pain out of proportion, a pain which is disproportionate to the apparent severity of the infection and extends beyond the visible borders of the skin change (site of infection)[243].

2. Crepitations under the skin, suggesting presence of gas in the subcutaneous layer (emphysema), which would normally not exist there [243].
3. Elevated levels of inflammatory markers such as C-reactive protein (CRP), lactate (suggesting tissue hypoxia), and procalcitonin [243].
4. Compromised vital signs, including low systolic blood pressure (less than 100 mmHg), elevated heart rate (over 100 bpm), low blood oxygen saturation, and fever [243].

If NSTI is suspected, a sample should be taken from the affected tissue for a rapid antigen test (strep-A test) to detect carbohydrate A antigen, which provides an immediate diagnosis if positive[244] . The time to diagnosis is critical, since NSTI caused by *S.pyogenes* can have a rapid response with patients entering septic-shock if the bacterial load and toxins produced damage the tissue[241]. Superantigens and necrotic debris exacerbate the host's inflammatory response, potentially leading to multi-organ failure [241]. Currently, sepsis is defined as a life-threatening organ-dysfunction caused by a detrimental response by the host to an infection, and septic shock is defined as a more severe form of sepsis where mortality rate is much higher. Monitoring a patient's vital signs is one method for assessing the severity of sepsis[241]. For example, a patient with normal blood pressure and heart rate without intravenous fluid support is at lower risk of sepsis. However, if the same patient, with the same skin infection, would have had elevated heart rate in combination with a low blood pressure the vital signs would indicate a more severe case [241]. A septic patient may present with elevated heart rate and low blood pressure, but may respond to intravenous fluid resuscitation. In contrast, patients in septic shock do not respond to fluid resuscitation and may require norepinephrine infusion (to elevate blood pressure through systemic vasoconstriction) [241]. Lung function can be monitored by assessing respiratory rate, blood oxygen saturation, and oxygen requirements. Kidney function can be monitored by measuring urine output per hour and serum creatinine levels. While no single test accurately diagnoses sepsis, blood tests revealing elevated CRP, lactate, decreased blood pH (suggestive of tissue hypoxia), and elevated organ-specific markers (such as ALT for liver or creatinine for kidney) can indicate a worsening clinical condition [241]. The problem of NSTI and subsequently the sepsis or septic shock that can happen, is the presence of high bacterial load which produces toxins that destroys the tissue and damages organs. The toxin activity, super-antigen release (more on this) combined with inflammatory debris from dead cells generates a strong immune response with excessive cytokine release resulting in sepsis and septic chock[245]. The cytokines, as we discussed in **Chapter 1**, can dilate the blood vessels which lowers blood pressure and the hypotension can induce damage to organ systems which are deprived of blood, and the negative spiral that happens can lead to the death of the patient.

Therefore, the goals of treatment are to reduce inflammation and eliminate the bacteria in addition to supportive assistance of the failing organs such as respirator care. Immediate surgical debridement is crucial; orthopedic surgeons remove infected tissue, sometimes requiring amputation, to prevent bacterial dissemination and reduce inflammatory debris. Early surgical intervention is essential to limit the inflammatory response and reduce bacterial burden [245]. Multiple debridement's are often necessary to achieve source control (reducing bacterial burden to a level where antibiotics can effectively eliminate the remaining bacteria).

The current standard of care in Sweden and most countries, in addition to surgery, involves adjunctive intravenous penicillin and clindamycin[246] . The penicillin is used to directly kill the Streptococcal bacteria while Clindamycin targets the ribosomal production of proteins, which is aimed at reducing the toxins which lead to necrosis of host-tissue (it also kills bacteria) [247,248] . Although not universally standard, intravenous immunoglobulin (IVIG) is used in the treatment of NSTI[247,249]. IVIG is a product of highly concentrated antibodies from more than 10.000 healthy donors, and in theory, would contain antibodies against toxins and other virulence factors expressed by the virulent GAS/strep and also be anti-inflammatory. However, clinical trials have been inconclusive on the benefit of IVIG treatment for NSTI[250]. In early 2011, CDC reported that 8% of invasive Streptococcal pyogenes isolates exhibit resistance to clindamycin, quite alarmingly, this figure has now increased to 30% in the span of 10 years[251]. Even when resistance is not an issue, the mortality rate remains high; in a recent study the 1-year mortality rate was 20%[242]. Therefore, there is a significant need for additional therapeutic agents that can improve bacterial clearance, reduce the host's inflammatory response, and prevent tissue destruction and organ damage. In this context, we will discuss the importance of Streptococcal virulence factors in NSTI and severe disease, as well as the potential of monoclonal antibodies as adjunctive therapy (with relevance to Paper II).

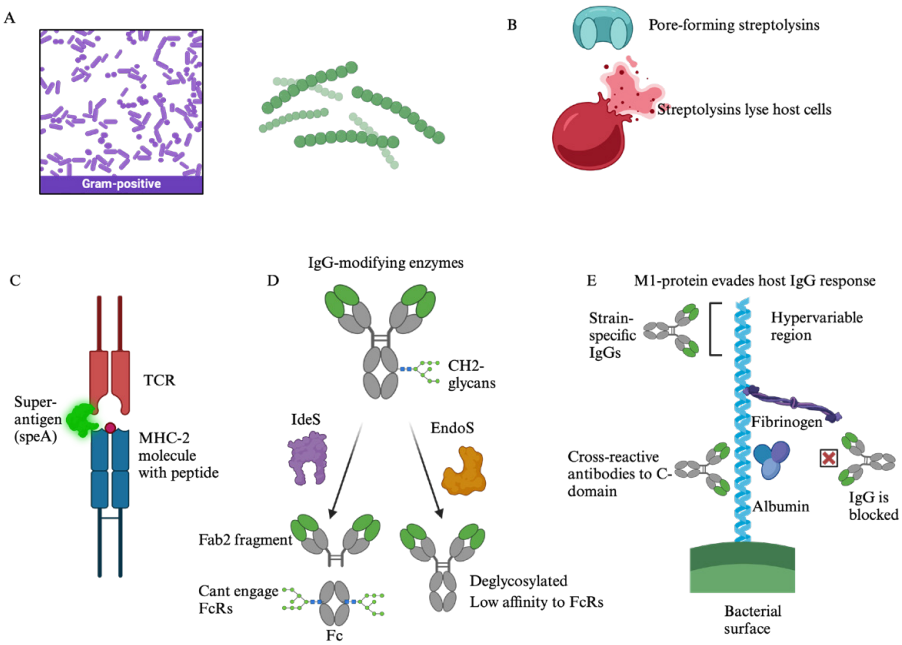

Figure. 13. Virulence factors of Group A streptococcus (GAS) / *Streptococcus pyogenes*.A Illustration of Gram staining, where streptococci appear Gram-positive (stained purple). The image on the right shows how GAS grows in chains. **B** Streptolysin O forms pores in host cell membranes, leading to cell lysis. **C** Superantigens crosslink T-cell receptors (TCR) with MHC class II molecules, resulting in aberrant T-cell activation and proliferation. **D** GAS secretes multiple IgG-modifying enzymes: IdeS cleaves IgG at the hinge region, inhibiting IgG-Fc receptor (FcR) recognition by removing the Fc domain, while EndoS removes IgG glycans, which are crucial for high-affinity interaction with FcRs. **E** The M1 protein is targeted by antibodies at both its hypervariable domain and a more conserved domain closer to the bacterial surface. The former leads to type-specific immunity, while the latter induces cross-reactive immunity. M1 protein also binds to host proteins such as fibrinogen and albumin shown to inhibit IgG opsonization, theorised to be due to sterically blocking opsonic epitopes. Created using BioRender.

VIRULENCE FACTORS IMPORTANT DURING ACUTE INFECTIONS FOLLOWING COLONIZATION

While *S. pyogenes* has many virulence factors crucial for establishing infection and evading pre-existing immune responses, several key factors are noteworthy when discussing acute NSTI, sepsis, and toxic shock syndrome. As discussed above, in NSTI, the bacterium produces toxins that destroy surrounding tissues, including skin layers, fascia, and muscles[230]. These toxins can eliminate macrophages, red blood cells, and other immune components. This is important for several reasons. Firstly, breaching the skin and deeper layers allows for wider and even systemic

spread of the infection Secondly, by directly eliminating effector cells of the innate and adaptive immune responses, bacterial growth is less hindered[230]. Additionally, it is worth noting that these toxins are more concentrated at the infection site. Thirdly, the release of cellular content from necrotic tissue promotes a strong inflammatory response, which may lead to sepsis, characterized by hypotension and organ failure, further weakening the host[242,243,247]. Thus, toxin production by GAS plays a critical role in disease progression, and we will discuss some noteworthy toxins below.

STREPTOLYSIN O, S AND NADASE

Streptolysin O is a 60-kilodalton protein that binds to cholesterol-enriched cellular membranes. Monomeric SLO clusters together and oligomerize and form a ring-shaped structure[230]. This structure, undergoing a conformational change, will then insert itself into the membrane and form a pore. Unfortunately for the host, SLO preferentially forms pores in neutrophils and macrophages but also affects other cell types such as erythrocytes (**Figure 13B**)[230]. Pore formation leads to cell death via unregulated ion influx, causing cellular swelling and lysis. The destruction of immune cells allows for evasion of phagocytosis (increased bacterial load) and allows for systemic spread (infection is not contained at the local site)[252]. Similarly, destruction of epithelial and endothelial cells by SLO reduces tissue integrity and further allows bacterial dissemination in the bloodstream. Interestingly, pore-formation facilitates bacterial invasion into non-phagocytic cells, creating a protective niche where the neutrophils and other phagocytes cannot reach the invader. Furthermore, SLO is known to synergize with other bacterial toxins such as NADase[253]. NADase, which exerts its cytotoxic effects intracellularly, can enter through the SLO-pores or via SLO-mediated translocation in a pore-independent manner[254]. Once inside the cell, NADase hydrolyzes NAD+, depleting ATP, leading to cellular death. Additionally, NADase prevents acidification of the phagolysosome in macrophages which allows intracellular survival in these cells by the bacterium[239]. Another noteworthy consequence of SLO is that its proinflammatory effects lead to microvascular thrombosis (in studies with rats), leading to further ischemia of the tissue which causes necrosis[255]. In a recent study by Tang et al. SLO was noted to facilitate the conversion of plasminogen to the active form plasmin, which lyze blood clots, potentially allowing systemic bacterial dissemination[256]. Overall, SLO and NADase play a critical role in GAS pathogenesis by promoting immune evasion, tissue destruction, and possibly enabling. systemic bacterial spread. Their synergistic effects and the clinical impact

they potentially have underscore the aggressive nature of invasive GAS infections such as NSTI and TSS.

Another streptolysin toxin, streptolysin S (SLS), is thought to contribute to the classical symptom of "pain out of proportion". It has been shown to directly activate pain-receptors in neurons (TRPV1+ noiceceptor neurons) which generates an unproportionate pain sensation in the patient[257]. The reason for this specific binding is believed to be that neutrophil recruitment is impaired by the release of a neuropeptide by the TRPV1+ neurons. In addition, SLS preferentially binds to erythrocytes (red blood cells) and keratinocytes, inducing osmotic changes leading to lysis[230]. Thus *Streptococcus pyogenes* can produce several toxins with varying specificities, complementing and enhancing each other.

STREPTOCOCCAL PYROGENIC EXOTOXINS- SPE

While NSTI is characterized by tissue necrosis caused by NADase, SLS, and SLO (among other virulence factors), a subset of patients exhibit an aberrant hyperactive T-cell response marked by excessive cytokine release. This condition, known as streptococcal toxic shock syndrome (STSS), is mediated by T-cell activation via streptococcal superantigens known as streptococcal pyrogenic exotoxins (SPEs)[258,259] .These interactions occur at the immunological synapse, where MHC molecules engage T-cell receptors (TCRs), as discussed in **Chapter 1 (Figure 13C)**.

To date, 13 superantigens produced by *Streptococcus pyogenes* have been identified, and among these[260] SpeA is considered a key mediator of streptococcal toxic shock syndrome and commonly associated with NSTI. These superantigens are small proteins, roughly between 22-28 kilodaltons in size. Interestingly, multiple superantigens are often produced simultaneously, with 3 to 6 different toxins typically expressed by a single bacterial isolate during infection. This phenomenon is believed to be due to the varying affinities that different toxins have to the numerous T-cell receptors (TCRs)[261]. SPEs possess the remarkable ability to cross-link MHC class II molecules with TCRs by binding to a conserved site on MHC class II and variable regions on the beta chain of TCRs. TCR binding generally occurs outside the peptide-recognition site and is independent of antigen processing[262]. Thus the binding of SPEs to MHC and TCR can directly activate CD4+ and CD8+ T-cells while also inducing cytokine release from the APCs. Furthermore, because numerous genes encode TCR variable domains, and VJ recombination generates diversity, it is not unsurprising that multiple toxins are produced to expand the range of targetable TCRs[263]. Similarly, variation in MHC class II alleles influence exotoxin binding. Additionally, different alleles of speA

significantly affect their binding affinity for MHC class II, with SpeA3 exhibiting the highest affinity in experimental studies. *In vivo*, T-cell activation induces pro-inflammatory cytokine production followed by initial T-cell proliferation and subsequent depletion[264]; this depletion is hypothesized to impair Immunoglobulin responses due to reduced CD4+ help[258]. Clinically, disease severity depends heavily on both the patient's genetic background and the combined phenotype of exotoxins produced. This variability makes it challenging to predict which patients will experience mild disease versus those requiring intensive care. Ultimately, streptococcal exotoxins drive T-cell activation by hijacking APC-T-cell interactions at the immunological synapse. This process induces a hyperinflammatory state that can result in sepsis and an inadequate immune response. The importance of SpeA activity has been demonstrated in murine models where pre-existing immunity induced through vaccination with a SpeA-mutant toxoid was shown to abolish infection[259].

EndoS and IdeS- targeting the humoral immune response

While SLO and SLS directly target the phagocytes by lysing them, other virulence factors that can interfere with the extracellular elimination of the pathogen by inhibiting IgG and complement opsonins. As discussed briefly in **Chapter 2,** the bacterium produces two enzymes called EndoS and IdeS which have high-specificity to human IgG[117,188]. EndoS, a bacterial glycan hydrolase, is a secreted enzyme that removes the glycans at the CH2 domain of human IgG's. As discussed in **Chapter 2,** the CH2-glycans are crucial for stable interaction between the IgG and its FcRs. Thus this glycan removal by EndoS reduces the affinity of the polyclonal IgG to their Fc-gamma receptors. In this manner, EndoS contributes to immune evasion by reducing Fc-mediated phagocytosis by neutrophils and macrophages. Similarly, IdeS enhances bacterial survival by escaping phagocytosis through cleavage of human IgG at the lower hinge domain, thereby abolishing interactions between the Fc and FcR (**Figure 13D**)[117,118,265]. It is worth noting that few studies have looked at *in vivo* activity of IdeS and EndoS from clinical settings in causes such as NSTI. However, Naegeli et al[266]. analyzed the polyclonal IgG in GAS-infected patients using mass spectrometry and observed IgG glycan hydrolysis at the local infection sites attributed to EndoS activity. The authors demonstrated EndoS activity *in vivo* using a murine model with a GAS mutant lacking EndoS and compared its outcomes to those of a wild-type (WT) isolate. Mice challenged with the WT GAS isolate exhibited significantly higher bacterial loads in various organs (e.g., spleen and skin), suggesting systemic spread compared to those infected with the EndoS mutant. However, it is worth keeping in mind that the design of these

animal experiments have great influence on the outcome measured. Toledo and Bratanis et al[267]. demonstrated that EndoS and IdeS activity varies greatly depending on the route of bacterial administration (e.g., subcutaneous or intraperitoneal injection). Thus, replicating what occurs in a heterogeneous patient population with diverse clinical isolates is highly challenging; experimental findings may lack direct relevance to clinical settings. This challenge is highlighted by a study analyzing neutralizing antibody titers against IdeS from patients with severe or mild GAS disease. Although the polyclonal mix of antibodies could effectively neutralize IdeS activity *in vitro*, the presence of neutralizing antibody titers did not influence the severity of disease- raising questions about this virulence factor for acute disease[265] . In another study, Karlsson et al[268] . analyzed IgG peptides from tonsillar swabs (tonsillitis) and skin samples (NSTI patients) from GAS infected patients to detect IgG cleavage by IdeS using mass spectrometry. They also analyzed IgG-rich environments such as tissue fluids and serum. The authors noted that in IgG-low environments (swab-material), IgG was efficiently cleaved by IdeS, while IgG-rich environments were not affected. IdeS is suggested to exhibit *in vivo* activity locally at sites with high bacterial loads and low IgG levels rather than within systemic circulation [268] . Taken together, GAS produces several virulence factors that specifically target human IgG to evade adaptive immune responses - highlighting their importance for anti-pathogen defense mechanisms.

M PROTEIN - THE HOLY GRAIL OF *STREPTOCOCCUS PYOGENES* RESEARCH

The M protein is a dimeric helical protein that forms a coiled-coil structure, protrudes from the bacterial surface, and is covalently linked to the peptidoglycan wall[233,269]. Electron microscopy images of GAS bacteria show that the M protein is predominantly expressed and covers nearly the entire bacterial surface. M protein has several critical functions for bacterial virulence, the most clinically relevant of which is inhibiting phagocytosis by neutrophils and macrophages[270]. Given its importance, vaccine design against GAS has been centered on this protein. However, the type of protein expressed by different clinical isolates varies significantly, and there is significant antigenic diversity in the N-terminal domain of the protein which happens to also generate an adaptive immune response (it has high immunogenicity)[232]. This region, therefore, is known as hypervariable domain and is used in serotyping strains. Strain typing is performed using PCR in a method called *emm*-typing Specific immunity can be generated against an infecting *emm* type through the production of antibodies targeting its hypervariable domain. This

concept, based on older historical research, is referred to as type-specific immune response[271]. A consequence of a strain-specific response is that it would possibly not result in a cross-strain reactive response against the other serotypes. With more than 200 different *emm* types[272], mounting a broad protective immune response remains challenging. More recent vaccine clinical trials have shown that different *emm* types can elicit opsonic antibodies that cross-react with non-vaccine serotypes[273]. Similarly, De Neergaard et al demonstrated that infecting strains from diverse *emm* types can raise cross-reactive opsonic antibodies against other dissimilar *emm* types[274]. Thus, in both infection and vaccine settings, it appears that cross-reactive, non-type-specific opsonic antibodies can be generated against diverse *emm* types. To better understand how monoclonal antibodies can be used as therapeutics targeting the M protein and how engineering can enhance these mAbs we will focus on the adaptive immune response to various M protein epitopes, its role in severe disease, and recent advances in monoclonal antibody development for this critical target.

STRUCTURE & FUNCTION OF THE M PROTEIN

The various *emm* types can be classified in different ways based on function, sequence, and structural architecture. One such classification is the *emm* pattern classification, which looks at sequence repeats in the overall protein. So far, there exists 5 patterns: A, B, C, D and E[269]. Of these, clinical isolates belonging to *emm* pattern A-C tend to cause pharyngeal infections, while D strains are more prominent in cutaneous infection, which illustrates that this classification system has good clinical relevance[275]. It is worth noting that apart from sequence variety which influences the humoral adaptive response, not all M proteins have the same effects on the host due to different structural differences. We will not discuss these differences; instead, we will focus on discussing the function and structure of *emm*1, which belongs to the A-C *emm* pattern. The focus on emm1 (M1 protein) is twofold, first it is the leading *emmt*ype responsible for invasive GAS disease worldwide[276]. It has been estimated that M1 and M3 expressing strains are responsible for 50% of all invasive disease[277]. Secondly, and most importantly, the focus of paper II is primarily on M1 expressing strains.

Like other emmtypes, *emm*1 expresses repeated motifs below the hypervariable region (named A, B, C, D and E repeats respectively). In the case of *emm1*, it has two B-repeat motifs and downstream of it (closer to the cell-wall) it has three C-repeats[269]. Each B repeat consists of 25 amino acids that are repeated 4 times, while each C repeat has 35 amino acids that are repeated 2 times. Not all *emmt*ypes contain

all these repeats, but the C-repeats are present in all *emmt*ypes. In the N-terminal domain of the M1 protein at the B1-B2 repeats, the bacteria can bind human fibrinogen and fibronectin[278]. Fibronectin binding by M protein has been previously linked to adhesion and invasion in epithelial cells, important for colonization and bacterial spread in tissues[279]. Fibrinogen binding to the M1 protein can lead to a complex structure which activates monocytes and neutrophils through activation of Beta-2 integrins[233]. Upon neutrophil activation by the M1-fibrinogen complex (which cannot happen by these proteins individually) heparin-binding protein is released by the cells which induce vasodilation of blood vessels, leading to vascular leakage and the patient can become septic (hypotension with organ-failure)[233,280]. The M1 protein has the ability of also binding human albumin at its C-terminal domain, which is believed to stabilize the coiled-coil structure. More interestingly binding of both fibrinogen and albumin to the N-terminal domain and C-terminal domain has been shown to inhibit the opsonic activity of antibodies binding to the B and C repeats between these domains. Antibodies binding to the hypervariable region did not suffer from this issue, suggesting that these serum proteins sterically block the binding of the B and C-repeat antibodies (**Figure 13E**)[281].

It is worth noting that other *emm*types can reduce complement activation by binding to negative complement regulators such as C4BP and Factor H, leading to less killing by complement opsonization[269]. Interestingly, M1 protein does not have this ability. Moreover, downstream of the B2 repeat is the S-region of the M1 protein, which can bind human IgG Fc at a micromolar affinity[280]. The M-protein bound IgGs can then be effectively cleaved by IdeS and their Fc-glycans hydrolyzed by EndoS which are secreted from the bacterial surface. Non-M protein specific antibodies can thus be rendered less efficient by the synergistic activity of M protein, IdeS, EndoS and other proteases (such as speB). Interestingly, in a recent study by Happonen et al[282], the authors noted that, in their MS-based approach of analyzing proteomic network of GAS-human serum/saliva interactions, IgG3 antibodies against M protein are dominantly responsible for opsonization of the bacteria. They also found that IdeS secretion by GAS is an efficient counter for this due to more susceptible cleavage of this subclass in the hinge domain by the protease. Thus there seems to be a delicate dance between human IgG, M protein and other GAS virulence factors such as IdeS.

DISCOVERY OF THE FIRST OPSONIC HUMAN MONOCLONAL ANTIBODY AGAINST THE M PROTEIN

Given the lack of efficacy of IVIG in improving survival rates in NSTI infections caused by *GAS*, researchers are exploring new therapeutic strategies. One promising avenue involves targeting the M protein, a key virulence factor and an important target for antibody-mediated opsonophagocytosis. In a study by Bahnan et al.[170], the investigators isolated IgG+ B-cells (CD19+/CD3-) reactive to the M1 protein using fluorescence-activated cell sorting (FACS) (see **Chapter 3** for details). These B-cells were obtained from a healthy convalescent donor who had successfully cleared a *GAS*-induced tonsillitis infection. However, the infecting strain and *emm* type were not identified. From these B-cells, the authors generated 10 monoclonal antibodies (mAbs), four of which exhibited strong reactivity to the M1 protein when tested using live bacteria expressing M1 protein (compared to a ΔM1 strain). These antibodies were designated Ab25, Ab26, Ab32, and Ab49.

Interestingly, one mAb clone, Ab25, demonstrated unique characteristics that set it apart from the others. Using cross-link mass spectrometry to analyze the epitopes targeted by these antibodies, it was found that Ab49 bound specifically to the B2-repeat region of the M1 protein. In contrast, Ab25 exhibited a dual-binding mode: it bound to the same epitope as Ab49 in the B2-repeat region but also targeted an additional epitope in the C-repeat region. This dual-binding mechanism, referred to as dual-Fab cis-binding, enabled Ab25 to bind two distinct epitopes on the same antigen. It is a natural bi-specific antibody in other words. This unique binding property translated into functional differences between the antibodies. While Ab49 could not mediate opsonophagocytic of heat-killed bacteria, Ab25 effectively promoted this process. The enhanced opsonic activity of Ab25 was attributed either to its bi-specific nature or to its ability to target the lower epitope in the C-repeat region, which Ab49 does not bind. Remarkably, Ab25 demonstrated potent opsonic activity that exceeded IVIG by several-fold: 10 μg/mL of Ab25 outperformed 1 mg/mL of IVIG in functional ADCP assays.

The protective potential of Ab25 was further demonstrated in a murine skin-infection model in the same study. In this model, mice were prophylactically treated with either PBS (control), IVIG (10 mg/mouse), or Ab25 (0.25 mg/mouse) prior to intraperitoneal challenge with a high inoculum of a hypervirulent M1-expressing strain (*AP1*). The authors assessed bacterial spread to distant organs and quantified bacterial loads to evaluate treatment efficacy. In the PBS group, all mice exhibited high bacterial loads in the spleen, kidneys, and liver (~100,000-300.000 CFU/g), indicating that their immune systems were unable to contain the infection. IVIG provided partial protection, preventing bacterial dissemination in 30–40% of mice

(roughly 4.000-20.000 CFU/g); however, most subjects in this group had bacterial loads comparable to those in the PBS group. In contrast, Ab25 provided superior protection: 50–70% of mice treated with Ab25 showed no detectable bacteria in their organs, and those with some dissemination exhibited significantly lower bacterial loads compared to PBS or IVIG-treated groups. Notably, this protective effect was achieved at a 40-fold lower dose than IVIG (2.5% of the IVIG dose). These results highlight Ab25's potential as a preclinical candidate for further development as a therapeutic agent for NSTI caused by *GAS*. Another promising feature of Ab25 is its specificity: it does not cross-react with human tissues and demonstrated cross-reactivity with all 13 *emm* types tested - despite significant sequence dissimilarities among these strains. This broad reactivity underscores its potential utility against diverse *GAS* strains. The pioneering work by Bahnan et al. has laid critical groundwork for advancing monoclonal antibody research targeting M protein. Paper II of this thesis builds upon this foundation and explores related avenues in greater detail.

Author comment on the chapter:

Streptococcus pyogenes imposes a significant disease burden, particularly in terms of the impact on life and quality of life during invasive infections. Even in non-fatal cases, patients often experience severe sequelae, including organ failure and amputation. New treatments are needed for this disease. Interestingly, analysis of patient outcomes suggests that the presence of endogenous M-protein-specific antibodies may protect against severe disease, potentially preventing its occurrence (making severe cases the tip of the iceberg, with many cases being managed by treatment and the patient's immune system). If this rationale holds true, the adjunctive use of M-protein-specific monoclonal antibodies (mAbs) could offer a promising new therapeutic approach for treating severe necrotizing soft tissue infections (NSTI) caused by Group A Streptococcus (GAS). However, can such therapeutics be enhanced for better effects through antibody engineering? This question is the primary focus of Paper II in this thesis and will be explored in greater detail.

CHAPTER VI
SARS-COV-2 - A CASE STUDY OF WHEN MODERN MEDICINE ROSE UP TO THE CHALLENGE

Introduction

At the end of 2019 and the beginning of 2020, reports were circulating that hospitals in China were seeing strange new cases of pneumonia[283]. In the region of Wuhan, cases were rapidly mounting. It was later discovered that a new virus, designated Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was the causative agent, and the disease was named Coronavirus Disease 2019 (COVID-19)[284]. This virus belongs to the coronavirus family, which is a known and common disease-causing virus. Common cold-viruses belong to this group, such as OC-43, NL-63 and HKU-1, which cause mild colds in humans by infecting the upper-respiratory tract. Coronaviruses can also infect other animals such as pangolins, camels, and bats, and through zoonotic transfer, they can spill over to humans[284]. As a matter of fact, in this decade, this has occurred at least two times prior to 2020, in the form of MERS (which came from camels)[285] and the SARS outbreak (emerged from bats)[286], which luckily never reached pandemic potential. This has been suggested to be due to patients dying from the disease or being largely severely ill combined with relatively low infectivity of these viral strains (compared to measles for instance)[284]. However, what would happen if they would have been more infectious and made the host critically ill at the same time? The answer, regrettably, we got in the form of "the COVID-19 pandemic, which has been estimated to have caused up to 7 million deaths as of March 2025, according to the WHO[287]. Furthermore, if one analyzes the number of excess deaths/mortality (the increase in number of deaths during this time period compared to the expected number of deaths as seen prior to the pandemic), the cumulative figure is estimated to be more than 25 million. Thus, it is most likely that there is a significant underestimation of total deaths caused by COVID-19 (due to underreporting of deaths). The origin of SARS-CoV-2 is a politically debated question as well as scientifically, with an animal-origin theory being one and the other is that it is an engineered virus created in a lab which, due to an accident, leaked (lab-leak theory)[288]. Irrespective of the origin, this virus has been highly successful in infecting human hosts leading to millions of hospitalizations, deaths and post-infection sequelae[289]. In this chapter we will briefly discuss the clinical manifestation, its virulence proteins, with a focus on the spike antigen, about the adaptive immune response and how monoclonal antibodies have been used to treat

this pathogen. We will emphasize the role of non-neutralizing Fc-functions in immune protection, of which all four papers in this thesis are focusing on.

OVID-19: FROM A CLINICIANS POINT OF VIEW

It is worth noting that, with new viral variants and as humoral immunity has been generated post-vaccination and infection, the clinical picture has been altered. However, in the beginning of the pandemic during the first year, patients who got infected were hospitalized to a much greater degree due to higher virulence of the virus combined with lack of preexisting immunity. SARS-CoV-2 utilizes a protein called Spike, which enables it to enter host cells expressing human angiotensin converting enzyme 2 receptor (hACE2R) **(Figure 14A-B)**[158]. hACE2R is expressed mostly in cells coating the epithelial line of the upper and lower respiratory tract, which allows for high infectivity of SARS-CoV-2 in the respiratory tract[290]. The clinical picture was thus dominated by symptoms from this area. While most patients remain asymptomatic or have mild disease in the form of fever, malaise, cough, dyspnea, confusion and myalgia. It was estimated early in the pandemic that 20% of infected become more severely ill with diffuse wide-spread infection in the lungs, pneumonia, with CT-scans showing changes which are called ground-glass (due to how it appears)[291–293]. Of these 20%, roughly a quarter needs oxygen substitution due to severe pneumonia, and extreme cases progress to something called acute respiratory distress syndrome (ARDS, **Figure 14C**). In one study[291], more than 50% of the patients with ARDS died due to the disease, in a cohort of patients from the Wuhan region when infected with the original strain in 2020. ARDS is defined as a hypoxic state (insufficient oxygen-carbon dioxide exchange in the alveoli) with bilateral infiltrates on CT scan (due to increased permeability of the blood vessels in the lungs). The risk of death in ARDS patients was associated with increasing age, male gender, comorbidities such as cardiovascular disease, chronic obstructive pulmonary disease (COPD) and metabolic syndrome (diabetes, obese, hypertension)[294]. Initially, treatment options were few, and patients were given oxygen and in some cases corticosteroid treatment which improved survival rates when patients had ARDS. Antiviral therapy in the form of Remdesivir, was also employed based on known *in vitro* activity against MERS, as well as a Malaria drug called Chloroquine [295]. Other treatment options were plasma therapy, where the idea was that passive transfer of IgGs (where some would be directed against the spike virulence protein) from survivors would improve clinical outcome of oxygen-requiring patients[296]. However, in all these cases, clinical trials did not display a clear

benefit except for possibly Remdesivir, which improved time to clinical improvement compared to control (not statistically significant) in a highly cited RCT[297].

Hope was lit in the form of monoclonal antibody therapeutics against the viral pathogen[296] . Using the techniques of monoclonal antibody discovery discussed in **Chapter 3,** researchers and companies could quickly generate high-affinity antibodies against the spike protein which neutralized the virus's ability to infect host cells. In 2021, U.S. Food and Drug Administration (FDA)(FDA) approved the use of three monoclonal antibody treatments, in the form of three treatments[296]:

1. Bamlanivimab (LY-CoV555) and Etesevimab (LY-CoV016) by Eli-lilly
2. Casirivimab (REGN10987) and imdevimab (REGN10933) By Regeneron
3. SotrovimAb by Vir Biotechnology and GlaxoSmithKline

These antibody treatments all showed reduction in the form of hospitalization and/or death, reduction of viral titers and milder symptoms compared to randomized controls for patients with mild-severe COVID. As an example, in the phase 3 randomized clinical trial assessing Bamlanivimab and Etesevimab, 10 patients out of 517 in the control group died while zero cases of death out of the 518 mAb treated group in addition to lower viral titers (measured at day 7)[298].

It is worth mentioning that COVID-19 is not a strict respiratory pathogen, it has been shown that it can infect all types of organs in the human body, including the brain, heart, kidney, liver and spleen, when analyzing tissues from deceased patients[291]. As a matter of fact, spike protein itself has been shown to deposit itself in various organs (bone, meninges and brain), with the clinical relevance of this remaining unclear, and will most likely be elucidated in the future. Importantly, COVID-19 has been associated with a broad-range of chronic symptoms which have occurred a few weeks post SARS-CoV-2 infection which are not attributed to any other disease- this is known as Long COVID[289]. Interestingly, acute COVID-19 infections have been associated with a hyperinflammatory immune response affecting multiple organs, known as Multisystem inflammatory syndrome in children (MIS-C)[299]. This hyperinflammatory state is distinct from the immune response in acute COVID-19 disease (severe disease) in adults in addition to other known hyperinflammatory conditions post-infection in children such as Kawasaki's disease (a form of vasculitis). Thus, although treatment for acute COVID-19 and prophylaxis by vaccination is crucial, several patients have complicated sequelae from infections such as in MIS-C and Long-Covid where much research is needed. This thesis focus is on the acute form of the disease cause by COVID-19 and subsequent sections will focus on the humoral adaptive response which leads to viral clearance and host protection, ending with

recent advances made in understanding on how Fc-mediated functions are protective and what this means for the future of the field.

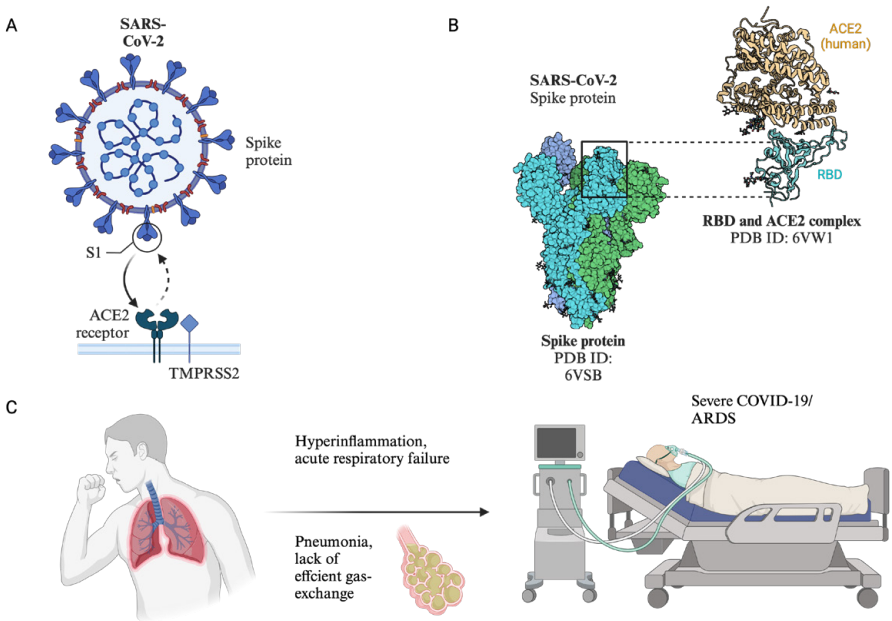

Figure 14. SARS-CoV-2 original strain caused lower respiratory disease. A. Illustration depicting the virion structure of SARS-CoV-2, with the spike protein highlighted. The top part of the spike protein, the S1 domain, contains the receptor-binding domain, which binds to the host ACE2 receptor **(B)**. The model of the spike protein in (**B**) is generated based on crystal structures from published work[300] , with accession numbers highlighted in the figure. After binding to human ACE2 receptors, a membrane-bound protease cleaves the spike protein, leading to a conformational shift in which the S2 domain protrudes into the host membrane, facilitating viral entry via membrane fusion. **C** depicts a patient infected by the virus in the lungs, leading to inefficient gas exchange in the alveoli. Many patients experienced acute respiratory failure due to a combination of hyperinflammatory immune responses and a lack of viral control in the lungs, requiring respiratory support in the ICU in severe COVID-19 cases. A subset of oxygen-dependent patients developed a condition called acute respiratory distress syndrome, which is associated with high mortality. Created using BioRender.

THE IMMUNE RESPONSE- THE BODY FIGHTS BACK

As discussed in **Chapter 1**, the innate immune system contains effector cells which can independently detect and fight of intruding invaders through pattern recognition receptors (for phagocytosis for instance), complement activation, NK-cell mediated killing by infected cells (independent of antibodies) and mobilize a pro-inflammatory response. PPRs are particularly important for intracellular detection

of foreign DNA, through the STING pathway. Such is the case with initial SARS-CoV-2 infection. One of the first lines of defense are the alveolar macrophages in the lungs, which can digest foreign pathogens and secrete proinflammatory cytokines to recruit more effector cells in addition to activate antiviral defenses (through interferon-gamma secretion to neighboring cells)[301]. However, it has been shown that SARS-CoV-2, like MERS-CoV and SARS-CoV-1, have several virulence factors such as ORF6 (an accessory protein)[302] which can downregulate expression of interferon-gamma induced genes by interfering with the intracellular STING-signaling pathway [301]. Thus, as hospitals were flooded in the initial first wave of the pandemic, the lack of preexisting immunity could not be compensated by the innate immune system for many patients, especially elderly who had preexisting conditions. As a matter of fact, severe COVID-19 disease has been associated with a dysregulated hyperinflammatory phenotype with potent proinflammatory cytokines correlated with inflammatory cell death (infected cells)[303]. Too potent inflammatory response can inhibit the adaptive immune response leading to impaired production and generation of humoral antibody response, which has been observed in COVID-19 patients [301]. Thus the clinical outcome is highly dependent on innate immune response, particularly the balance in cytokine mobilization which can be influenced by host genetic factors, preexisting conditions and the pathogens virulence proteins.

While the innate immunity mobilizes against the virus, the adaptive immune response is being generated in the germinal centers of lymphoid organs as discussed in **Chapter 1**. The main protective immune response is generated by T-cells recognizing epitopes on the spike protein and polyclonal antibody response by B-cells against the same target. Development of anti-spike CD4+ and CD8+ T-cells has been linked to viral clearance and mild disease[304], while lymphocytopenia in COVID-19 patients have poor clinical outcome[292], suggesting a crucial role for B and T-cells. In patients undergoing B-cell depleting therapy, CD8+ T-cell has been correlated with improved clinical outcome as well, potentially compensating for the lack of humoral immune response in these immunocompromised patients[305]. However it is worth noting that excessive CD8+ T-cell activation has also been linked to poor outcome[306], which most likely reflects the high viral load in these patients where the immune system has failed to clear the infection [306]. T-cell immune response is maintained to counter a second reinfection of SARS-CoV-2, suggesting a more durable response than the humoral one where titers drop after a few months post-infection or vaccination[307] .

CORRELATE OF PROTECTION- IMPORTANCE OF MEASURING THE "PROTECTIVE FUNCTION"

As discussed in **Chapter 1**, the humoral adaptive immune response is dependent on efficient T-cell help for somatic hypermutation, class-switching, antibody production by plasma cells and memory-formation[308]. The antibody response against SARS-CoV-2 has been shown to be intricately linked to protection in clinical trials when accessing vaccine efficacy but also post natural infection[309]. The correlation of protection has been linked to both neutralizing anti-spike titers and overall anti-spike titers[310]. However, it is worth noting that using neutralizing titers of anti-spike antibodies could potentially mask other protective immune functions which these neutralizing antibodies can elicit[311]. As a matter of fact, antigen-binding antibodies can be neutralizing, and can be opsonic, and neutralizing antibodies can thus be both opsonic and neutralizing[130]. Hence, when measuring neutralizing function in serum without measuring other antibody functions, the correlation of protection might be given too much weight to neutralizing function compared to ADCP, ADCC and other antibody based effector functions. More work is emerging showing that these functions[130,303,312,313] are protective in humans and in animal models, which stresses the importance of using these metrics as well in a broader correlate of protection determinants.

THE RBD IS IMMUNODOMINANT FOR BOTH NEUTRALIZING AND NON-NEUTRALIZING ANTIBODIES

One of the key components of the immune response against SARS-CoV-2 is the generation of antibodies that prevent viral entry into host-cells. As discussed in **Chapter 2**, these neutralizing antibodies differ in their epitopes where some target the receptor-binding domain (RBD) and others target non-RBD sites (such as NTD and the S2-domain)[159]. Interestingly, independent research groups have discovered that the germline-family of the V-gene (such as IGHV3-53) is a key determinant for neutralizing mechanism against the RBD domain, where little to no SHM was required to efficiently neutralize the wild-type virus[314]. Similar findings have been shown to be the case for antibodies against the S2 domain, where germline mAbs (without SHM) could efficiently neutralize a diverse set of Betacoronavirus (SARS, MERS, SARS-CoV-2)[87]. However, with the emergence of SARS-CoV-2 mutants such as Omicron, which contained 15 new mutations in the RBD at epitopes important for neutralization, many neutralizing antibodies lost their function[315]. Similarly, many clinically approved antibodies were rendered obsolete by the emerging sub variants from Omicron. Interestingly, these epitopes that were affected by mutations did not impact the T-cell response[316], which proved to be more durable than the B-cell

response against these variants[317]. It was later shown that the viral evolution was driven towards increasing its affinity to the human ACE2 receptor[318] while also evading the humoral immune response[319]. Consequently, with the advent of mRNA technology for vaccine design, the vaccine boosters employed during the later parts of the pandemic could be tailored to the dominating strain circulating[320]. However, due to the presence of preexisting memory B cells, booster vaccinations did not significantly elevate neutralizing antibody titers against the updated spike protein due to a phenomenon known as original antigenic sin[321]. Thus, with the emergence of new mutations in the spike protein, and due to preexisting immunity against the previous infecting strain and vaccination, the humoral immune response is more limited in generating new neutralizing antibodies against novel spike variants[319]. Additionally, with each new variant, the efficacy of neutralizing antibody titers has declined, while viral infectivity has increased[322]. Notably though, the newer variants post-Omicron have higher tropism for the upper-respiratory tract, reduced fusogenicity (promotion of host cells fusing together leading to tissue damage), resulting in more cold-like symptoms rather than pneumonia-type disease as with the WT strain [322]. It is also worth noting that, while neutralizing response is lower than against WT strain, T-cell response remains robust[316] and other non-neutralizing antibody responses have been shown to mediate protection in humans[303]. The evidence for these Fc-mediated functions will be discussed in the next section.

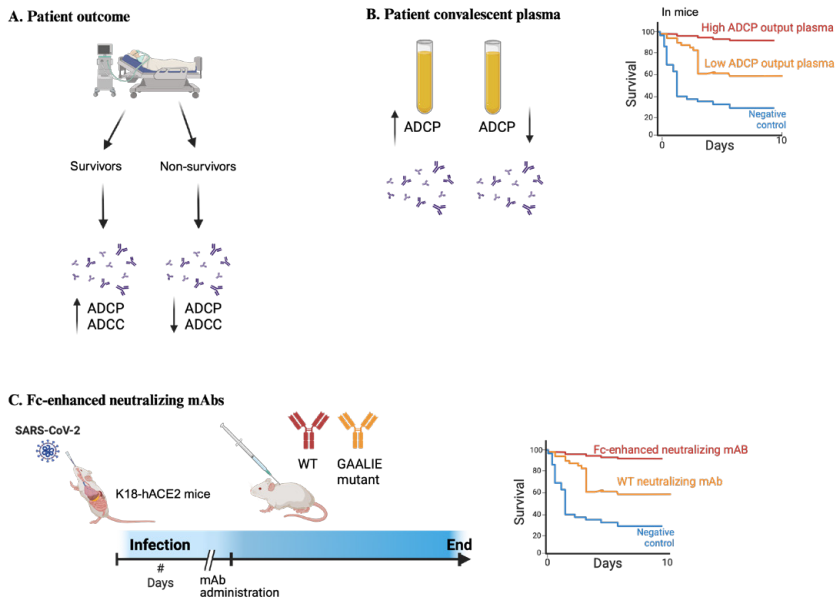

Figure 15. Non-neutralizing effector functions correlate with increased survival in the clinic and in animal models. A Illustration showing that clinical outcomes correlate with the Fc functionality of the polyclonal IgG response to the spike protein, where strong antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) functions are associated with survival. **B** Plasma transfer therapy studies indicate that convalescent plasma with stronger ADCP and ADCC functions—despite having the same neutralizing titers—provides better protection against lethal infection in K18-hACE2 mice. **C** Similarly, Fc-enhancing mutations in neutralizing antibodies correlate with improved survival in K18-hACE2 murine models.Created using BioRender. **Note:** Adapted with major modifications from an original figure by Izadi and Nordenfelt published in *Trends in Immunology*[130] .

Protective Non-Neutralizing Fc Functions: The Overlooked Mechanism in SARS-CoV-2 Immunity

As the mutations accumulated in the spike RBD and NTD, sites where almost all neutralizing antibodies targets, serum neutralizing titers dropped markedly[323] . For example, in a population that had received three vaccine doses based on the WT strain, neutralization of the XBB variant was 71-fold lower compared to the original strain[319]. However, it was noted that the polyclonal antibody response could still maintain Fc-mediated functions such as ADCC and ADCP, meaning that most of the antibody functions were maintained despite a significant drop in neutralizing ability[324].These antibody responses were generated in both natural and vaccine-induced immunity. The clinical relevance of these functions has been elucidated in an excellent study by Zohar et al[303], where they analyzed IgG response from

survivors and non-survivors. In cases where the clinical outcome was positive, the anti-spike IgG response correlated with strong FcR-binding and function. Interestingly, non-survivors had a markedly worse Fc-functional output in their anti-spike response (**Figure 15A**). Neutralization titers did not differ among the groups, suggesting that for viral control and clearance (after infection has taken place) Fc-functions are crucial for survival. This study was supported by Yu et al[325], where they observation that, in vaccinated rhesus macaques, reduction in viral load in the lower-respiratory tract correlated more strongly with ADCP and ADCD function by RBD-antibodies compared to neutralizing titers. These findings suggested that both Fab and Fc-function are essential for viral control. These studies have been further supported by *in vivo* data from animal work where neutralizing monoclonal antibodies with enhanced Fc-function (by GAALIE, GASDALIE mutations) protect much better than the WT mAb in K18-hACE2 and golden-hamster models. This was discussed in **Chapter 4** in detail (see study by Winkler and Yamin et al, **Figure 15B)**[157,326]. Interestingly, in those studies, the Fc-enhanced antibodies protected better in a therapeutic context, further reinforcing that Fc-function is crucial for viral control post-infection while neutralizing ability is perhaps more important for prophylaxis. However, as we discussed above, neutralizing antibodies can mediate both Fab and Fc-function, and thus measuring only neutralizing titers can possibly omit the protective correlation of their Fc-function. Non-neutralizing Fc-functions have also been shown to be important in passive-plasma transfer studies, where plasma with greater Fc-functionality offered greater protection than those with lower in animal models (**Figure 15C**)[313]. Finally, in a study led by Bahnan et al[171], the first instance of a non-neutralizing antibody, targeting the NTD domain, was shown to protect K18-hACE2 mice in a comparable fashion as a potent RBD-neutralizing antibody in a therapeutic model. These studies have together demonstrated that Fc-function is important for protection against emerging Betacoronavirus, and vaccine design and therapeutic monoclonal antibody development would benefit by taking this into account. Several independent studies[327–329], in addition to Paper I of this thesis[330], have demonstrated that non-neutralizing antibodies are highly protective in animal models against original WT strain. In Studies I-IV of this thesis, we aim to address this issue, with promising results in understanding how to generate an optimal Fc-functional response.

Author Comments on the Chapter: Towards Broadly Betacoronavirus-Protective mAbs?

The SARS-CoV-2 pandemic effectively demonstrated how modern medicine can rapidly adapt to emerging pathogens. In the early stages, no vaccines, therapeutics, or strong pre-existing immunity were available. By isolating B cells from survivors, researchers successfully generated protective monoclonal antibodies (mAbs) with a primary focus on virus neutralization. However, the high mutation rate of the spike protein has proven challenging, rendering all previously approved mAbs obsolete. Although two new mAb therapeutics have recently received approval, their ability to target conserved, mutation-resistant epitopes remains uncertain SARS-CoV-2 is just one of many Betacoronaviruses capable of causing severe disease in humans. Given the high probability of another pandemic in the next 10 to 30 years, the development of broadly protective mAbs is imperative. However, conserved neutralizing epitopes across Betacoronaviruses such as SARS, MERS, and HKU1 are scarce, making this a significant challenge. Recent studies, including Bahnan et al., suggest that non-neutralizing epitopes can also confer protection through Fc-mediated mechanisms. This raises a crucial question: should future therapeutic strategies prioritize broadly protective non-neutralizing pan-Betacoronavirus mAbs, rather than focusing solely on neutralization While neutralizing antibodies have historically been the primary focus of antiviral mAb development, the emerging evidence for Fc-function-mediated protection warrants further exploration. A shift in strategy—emphasizing non-neutralizing yet functionally protective mAbs could provide a more robust, long-term defense against future Betacoronavirus outbreaks.

"By failing to prepare, you are preparing to fail." - Benjamin Franklin

CHAPTER VII
PRESENT INVESTIGATION

AIM

Originally, this thesis focused on monoclonal antibodies targeting bacterial pathogens, with particular emphasis on *Streptococcus pyogenes*. However, with the emergence of SARS-CoV-2, which caused the COVID-19 pandemic, the focus shifted to Betacoronaviruses. SARS-CoV-2 presented a unique opportunity to study the host antibody response to this novel virus specifically, the protective functions of these antibodies and how they can be enhanced through Fc-engineering. The overall aim of this thesis was to elucidate the factors that make an antibody efficient at mediating immune functions, particularly Fc-mediated phagocytosis.

Given the distinct features of human IgG subclasses, we began by engineering the constant domain of IgG1 to that of IgG2-4 (Paper II). Additionally, due to challenges in the production and development of IgG3 such as aggregation and the existence of multiple allotypes its potential for clinical use has largely been overlooked. Therefore, one objective of this thesis was to investigate the Fc-mediated functionality of IgG3 in the context of both SARS-CoV-2 and *Streptococcus pyogenes* infections.

The unexpected importance of non-neutralizing SARS-CoV-2 monoclonal antibodies for immune protection led to further questions about whether they maintain efficacy against highly mutated SARS-CoV-2 variants. This formed the basis for the aim of Paper III. Finally, the creation of a hybrid IgG1-IgG3 antibody in Paper II, combined with the importance of Fc-function, prompted us to explore whether a potent opsonic monoclonal antibody with broad Betacoronavirus reactivity could be generated. This was the goal of Paper IV.

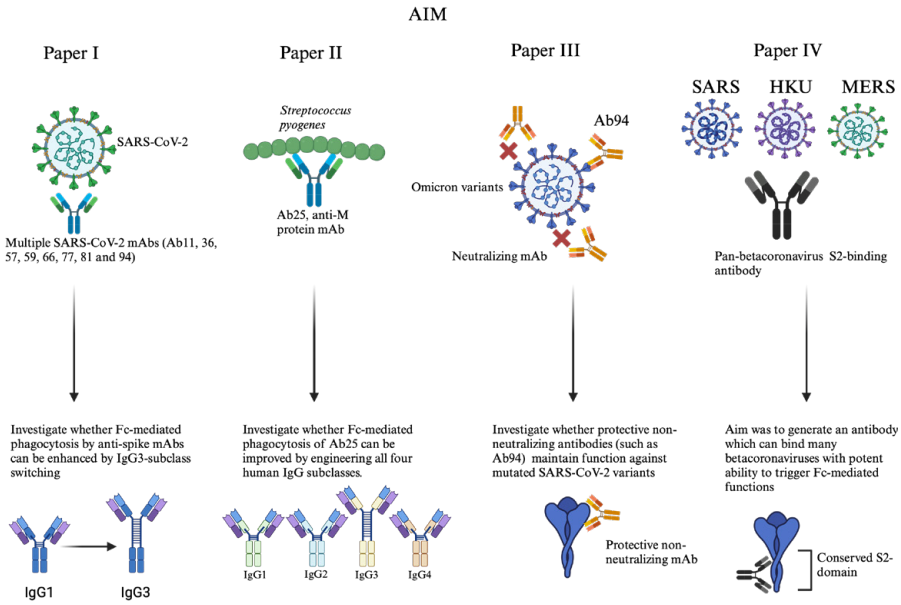

Figure 16. Overall aim of respective Papers (I-IV). Pathogen and monoclonal antibody is highlighted for each paper, with the research aim highlighted below in text. For Paper IV, the purpose was to discover a broadly binding S2-mAb, and therefore no specific mAb was outlined. Created using biorender.

Taken together, the four papers in this thesis explore how to generate a potent opsonic monoclonal antibody against two distinct pathogens while investigating the key factors that make an antibody functional. These studies complement and extend one another. The specific aims of each paper are as follows:

Paper I: Investigate whether subclass-switching IgG1 monoclonal antibodies targeting the spike protein can improve or reduce Fc-mediated phagocytosis and complement activation. Additionally, evaluate the protective role of these monoclonal antibodies in animal models against this novel threat.

Paper II: Generate all four IgG subclasses of Ab25, the first protective monoclonal antibody against the M protein of *Streptococcus pyogenes*. The goal was to assess whether we could create a more potent version of this promising preclinical candidate by starting with the original subclasses and subsequently performing further engineering.

Paper III: Based on findings from Paper I, where non-neutralizing anti-NTD and anti-RBD monoclonal antibodies were protective against lethal SARS-CoV-2

infection in animal models, we aimed to evaluate the durability of these antibodies against rapidly mutating spike protein epitopes that rendered clinically approved neutralizing monoclonal antibodies obsolete. Additionally, we explored the role of non-neutralizing antibodies in conferring protection against the Wuhan strain, as seen in Paper I.

Paper IV: Building on the findings of Papers I-III, we aimed to generate a potent opsonic antibody targeting several Betacoronaviruses to determine whether such antibodies could serve as future therapeutics in the event of new pandemics.

In summary, the aim of this thesis evolved as new information was acquired. The four studies build upon one another, contributing to our understanding of how to generate effective monoclonal antibodies against two distinct pathogens while aiming to explore what key factors make an antibody functional.

General Methodological Considerations

Monoclonal antibody production

In this thesis, monoclonal antibodies were widely used. There are several considerations in terms of method of production (cell-line, purification) and cloning strategies for engineering. The monoclonal antibodies used in paper I-IV were all produced in house by the methods described in **Chapter 3.** The plasmids that were generated were transfected in a human derived cell-line called Human embryonic kidney cells 293T (HEK293)[331]. These cells have been widely used in industrial settings in addition to academic research. The benefit of using a mammalian cell line system, and a human at that, is that it would more likely produce post-translational modifications comparable to the existing ones in the human donor (that the mAb was derived from)[332]. A competition to HEK293 cells is the CHO cell-line which is also widely used. However, these cells are derived from hamsters (chinese hamster ovarian cells) and could thus be less suitable for human monoclonal antibody expression, due to possible alterations in the post-translational modifications compared to those in the human donor[333]. Furthermore, previous work by Bahnan et al[170,171], used the HEK293T and Expi293 cells, and to make the data comparable across studies, similar cell-lines had to be used. These two considerations resulted in the production of the engineered mAbs in the HEK293T cell line and later Expi293 cells (for *in vivo* large scale production).

CLONING METHODS

The generation of Fc-engineered monoclonal antibodies in this thesis focused on modifying large segments of the antibody's constant domain, rather than altering glycans in the CH2 region or introducing point mutations. As a result, glycan-modifying enzymes and cloning strategies for point mutations were not suitable. While restriction digestion could have been an alternative given the availability of enzymes that can cut constant domain sequences at desired sites to facilitate sequence insertion, the chosen method was HIFI-DNA assembly (or Gibson assembly)[334,335]. This approach involves designing primers that amplify the sequence of interest with overhangs complementary to the vector. Enzymes in the HIFI-DNA assembly kit then anneal these complementary fragments. The advantage of this method lies in its simplicity, as primers can be tailored to target specific gene segments, enabling the creation of hybrid IgG constructs in Paper II. Once the construct is generated, the same method can be used to insert the variable domain into the newly engineered constant domain, allowing for the generation of additional Fc-engineered clones, as demonstrated in Papers I and II.

THP1-CELLS AND PAN-METHOD TO STUDY PHAGOCYTOSIS

In this thesis, the monoclonal antibodies against *Streptococcus pyogenes* and SARS-CoV-2 were characterized based on their ability to promote phagocytosis. A cell-line called THP-1[336] was used as a standardized cell-line to assess this function, for several reasons. One, this cell-line is easy to culture and exhibits acceptable batch to batch variability and can be used for several weeks. Secondly, THP-1 cells express human Fc-gamma receptors and have been widely used for assessment of antibody functionality in terms of ADCP[337,338]. Thirdly, previous work in the lab had characterized monoclonal antibodies used in this thesis by using THP-1 cells, and to make the data comparable we opted to use these cells as well[170,171,274,339]. However, it is crucial to also include the human donor-variability, and to address this we isolated human neutrophils and monocytes from healthy donors to complement the THP1-data (Paper I-II). Although we saw greater variability between the different donors using primary cells, we did not see any differences in results or trends when comparing between THP-1 data and primary cells.

A great deal of effort has been devoted to by Dr Neergaard in the lab in developing a robust and reliable assay to assess phagocytosis. This method, called Persistent-associated normalization (PAN)[339], utilizes a flow-cytometer as the detection tool to characterize how each cell is engaging the pathogen, or in the case of SARS-CoV-2, streptavidin beads coated with spike protein. By staining the prey (bacteria/beads)

with a pH sensitive dye and a pH-stable dye, the interaction between cells and preys can easily be characterized (once the prey is internalized in the cell in the phagolysosome the lower pH will activate the pH-sensitive dye). Furthermore, by considering the ratio between the prey and phagocyte, a factor called multiplicity of prey (MOP), the experiments can be more easily standardized and compared across experiments[340] . Flow-cytometry is itself a powerful tool to characterize many cells (in the order of thousands of cells) in a short period of time, as compared to traditional microscopy methods. Alternative assays for functional outcome would be opsonophagocytic killing assays[341], but those have been historically difficult to reproduce in the case of GAS and only study the outcome of the phagocytosis and not the interaction itself, as in the PAN method (where you can classify cells in internalizing prey, surface-bound prey or non-associating with prey). The PAN-method[339,340] has been instrumental in producing reproducible results in terms of differences between monoclonal antibodies, for both primary cells and THP-1 based experiments.

Affinity measurements

Affinity measurements were mostly done by using Surface-plasmon resonance which is the golden-standard to determine the molecular affinity between a drug and its ligand, especially in the antibody field[342]. Similarly, using ELISA is widely employed to calculate affinity. In paper I, III-IV this was done. In paper I, we used three methods (ELISA, SPR and flow-based assay) to analyze the binding of our IgG3 engineered mAbs against the spike protein, and compared the results to our IgG1 versions. This was done to observe if any trend was consistently seen by us when using different modalities. For paper II, when analyzing the affinity to M1 protein, only a flow-based assay was used because M protein is known to alter its structure depending on its attachment to the bacterial wall, temperature and other factors[269]. Thus studying it in its native expression on live bacteria provides the most relevant contextual data.

Animal models

In the beginning of the COVID-19 pandemic, several novel animal models were created to study the pathogenesis of the disease but also to assess potential therapeutic benefit of drugs such as neutralizing monoclonal antibodies. One of the most widely employed was the K18-hACE2 murine model, which, upon challenge of a sufficient inoculation of live virus, could mimic severe disease in COVID-19[343].

While the golden syrian-hamster model[344] is also a suitable candidate, the K18-hACE2 model is suitable for mimicking severe disease. However, for monoclonal antibodies, using non-humanized animal models, concerning the Fc-receptor expression, makes it difficult to assess the benefit of different human subclass-versions of mAbs. Although, if the model can capture the overall benefit of the non-neutralizing antibody response, questions regarding the biological importance of these functions for immune defense can be addressed. In paper I and III this was the main objective of the animal experiments using the K18-hACE model with a virulent SARS-CoV-2 strain in paper I (Wuhan) and a mutated variant (JN.1).

On a similar note, for Streptococcal challenge models BALB/6 mice were used to create a model for severe streptococcal disease using the virulent strain AP1. The infection route has been shown to be crucial for host-pathogen interactions, where subcutaneous administration has shown to upregulate critical virulence factors in the form of IdeS and EndoS (discussed in **Chapter 5**)[267]. Hence, the model considers these critical virulence factors which would occur in a real NSTI infection. However, the model suffers from the use of non-humanized murine animals in terms of Fc-expression, which limits the transferability of the findings to some degree. Animal models have now been created in the last decade which express human Fc-receptors on murine phagocytes, or even express human phagocytes (human PBMC engrafted mice) with human Fc-receptors to address this[162,345]. The clinical transferability of these models are also not understood, and there is a challenge in comparing murine animal models to a human setting regardless. Nevertheless, at the time of these studies they were not widely available nor affordable, but future work could benefit from their use.

ETHICAL CONSIDERATIONS

There are two clear ethical aspects which had to be considered for the projects in this thesis. Firstly, when characterizing the monoclonal antibodies in this work, we utilized human neutrophils and monocytes which were purified from human blood from healthy donors. These donors had been given written and oral consent before participating. It is important that the procedure is as risk-free and pain-free as possible and that the use of these cells are used in the right indication. Furthermore, these ethical considerations need to be approved by a local ethical committee. For our experiments we had ethical approval granted by the regional Swedish ethical review authority in Lund (Etikprovningsnamnden Lund, 2015/01801). Similarly for animal experiments, ethical approval had to be granted, where approval was given for the streptococcal animal experiments (Paper II) by the local Malmö/Lund Institutional Animal care and Use Committee (ethical permit number 03681-2019). As for the COVID-19 experiments, ethical approval had been granted by the Regional animal experimental ethics committee in Stockholm (16765–2020). It is important to consider, what was previously the 3Rs (Replace, reduce and refine), the 5 Rs[346] (replace, reduce, refine, reuse, and rehabilitate) when designing the animal experiments which we tried to do. The considerations that we particularly focused on is that we only perform these experiments when needed, have as few animals as possible but enough to see a clinically relevant and statistically powered effect (that is not have too few animals and must redo the experiment). In the streptococcal model, the animal experiments were short, only 30 hours, since we could acquire enough results from the murine organs (looking at bacterial load) rather than continuing the experiment until all mice died naturally (survival-curves), and needlessly prolonging their suffering. Thus ethical considerations did have a large impact on the way the animal experiments were designed and implemented.

RESULTS

PAPER I

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

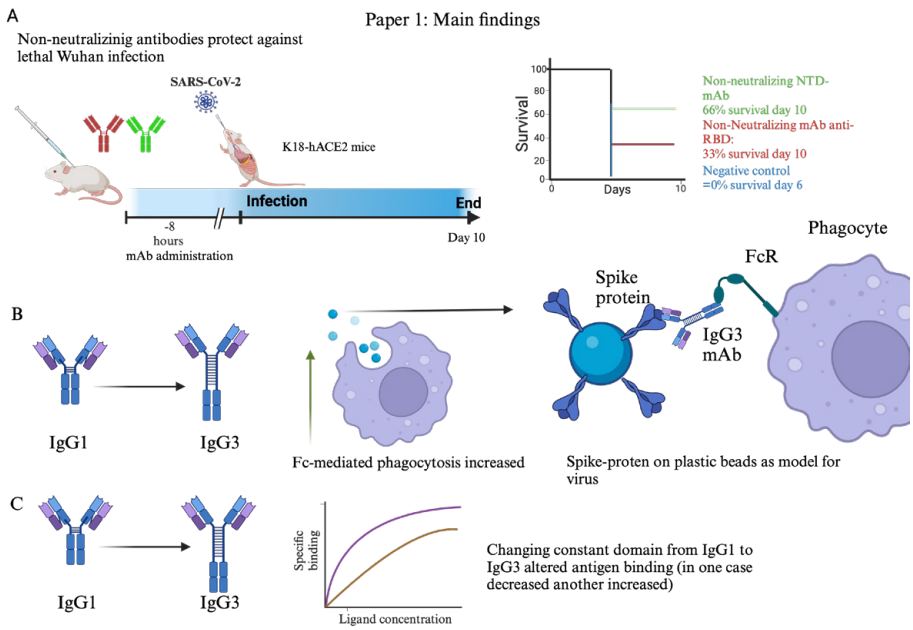

Figure 17. Overview of the main results in Paper I. A. Non-neutralizing RBD mAb Ab81 and non-neutralizing anti-NTD mAb Ab94 strongly increase survival in K18-hACE2 mice when challenged with a lethal inoculum of Wuhan virus (100.000 PFU). **B** Changing subclass from IgG1 to IgG3, in 7 out of 8 mabs, potently increased Fc-mediated phagocytosis by several-fold, and cocktail use of IgG3 mAbs had the highest ADCP and ADNP function. **C** Altering the IgG constant domain influenced the binding properties of two unique clones- challenging the notion of constant domain and variable domain independence. Created using biorender.

Given SARS-CoV-2's impact on society in terms of hospitalization and death tools, it became crucial to study which features of the immune response result in protection against death and severe disease. The results of such a research can help generate new therapeutics and prophylaxis in terms of vaccines that can elicit such a response. Monoclonal antibodies was one key therapeutic for acute disease but also prophylaxis for immunocompromised patients. Thus studying the protective

immune response of monoclonal antibodies became a promising starting point to understand how we can develop better therapeutics for this disease and for future outbreaks of the Betacoronavirus family, and possibly other viral pathogens such as influenza. Almost all focus on monoclonal antibodies centered around the spike protein antigen, and of those antibodies almost all tested in animal models have been neutralizing antibodies, either against the RBD or NTD site. In a study by Bahnan et al[171], the authors showed that mice treated with either a neutralizing RBD or a non-neutralizing NTD mAb (called Ab94) maintained body weight and improved survival compared to the negative control. This study, published in January 2022, highlighted the first case were non-neutralizing Fc-functions of a monoclonal antibody being protective against severe disease in animal models. This finding was the starting point for Paper I.

Given IgG3 subclass status in the literature as being proinflammatory with IgG1, compared to IgG2 and IgG4, engineering from the original IgG1 subclass to it became a natural starting point to examine how potentially protective Fc-function can be modulated for improved therapeutic efficacy. We engineered 8 monoclonal antibodies (Ab11, 36, 57, 59, 66, 77, 81 and 94) from IgG1 to IgG3 then assessed their binding to the spike antigen or their respective epitopes (RBD or NTD) and thereafter analyzed their Fc-mediated function by using the PAN-assay[339] with THP-1 cells. Interestingly, when changing the subclass for two clones (Ab11 and Ab57) we observed a significant alteration of binding avidity to the spike protein. These results suggested that changing the constant domain of an antibody can influence the binding properties governed by the variable domain- challenging the dogma that these domains are fully independent (as discussed in **Chapter 2**). These findings were verified with three independent assays (ELISA, Cytoflex based assay and SPR) done by 3 independent scientists suggesting it is not dependent on a technical issue. Furthermore, when comparing the 8 IgG1 versions with the newly engineered IgG3 versions, 7 out of 8 IgG3 mAbs vastly outperformed their original counterparts in mediating Fc-mediated phagocytosis by THP-1 cells. Ab94 IgG3 stood out as the best candidate closely followed by an RBD mAb Ab81 IgG3. Interestingly, when using the mAbs in a cocktail mix, at the same dose, the cocktail IgG3 mAbs generated the strongest Fc-mediated response exceeding even the best single mAb (Ab94 IgG3). This was also seen for Neutrophil mediated phagocytosis and complement activation. The findings were verified with neutrophils in a microscopy-based assay as well, suggesting this is a consistent phenotype of the IgG3 mAbs. Finally, the protective benefit of non-neutralizing antibodies were assessed in an *in vivo* experiment with K18-hACE2 murine model with authentic Wuhan virus. While most experiments use a dose of 1000-10.000 PFU of the virus[157,326,329], we used 100.000 as to see if the mAbs could improve survival. While

100% of mice in the control group died by day 6, the non-neutralizing IgG1 and IgG3 group showed improved survival and one group (Ab94 IgG1 treated mice) had more than 66% survivors at day 10 (end of the experiment). In addition, the first reported protective non-neutralizing RBD mAb (Ab81 IgG3) was reported in this study (33% survival at day 10 vs 0% survival at day 6 for control). These results strongly suggest that non-neutralizing antibodies are protective against severe disease and that these functions can be enhanced by IgG3 engineering, however the comparison between human subclasses in a non-humanized murine model limits the transferability in a human setting.

PAPER II

The hinge-engineered IgG1-IgG3 hybrid subclass IgGh47 potently enhances Fc-mediated function of anti-streptococcal and SARS-CoV-2 antibodies

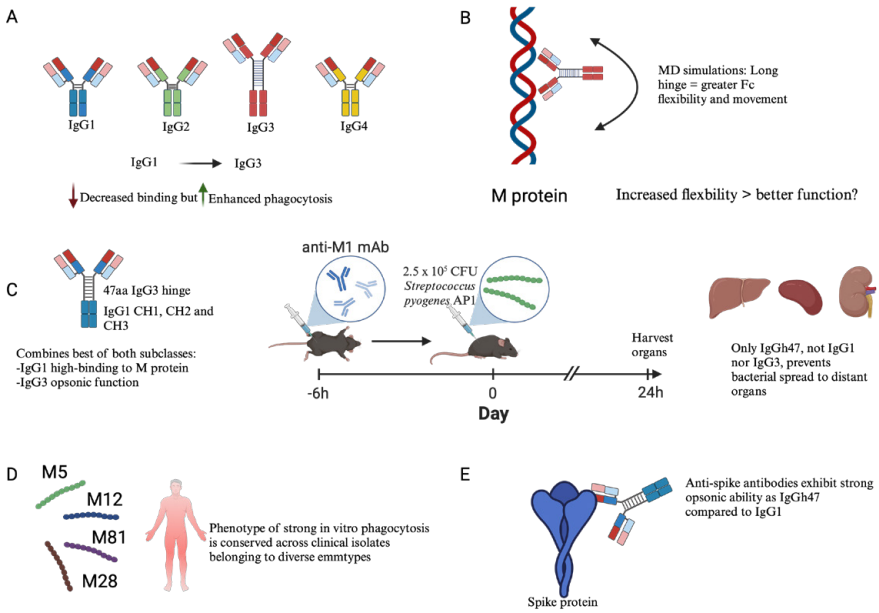

Figure 18. Overview of results in paper II. A. Generation of all 4 human IgG subclasses of Ab25. IgG3 variant had markedly reduced binding to M protein expressing bacteria while also having several-fold higher ADCP ability. **B** Molecular dynamics simulations revealed that IgG3 version has much greater movement in its Fc domain in 3D space relative to the antigen- compared to original IgG1. Attributed to its 62 aa long hinge. **C** A hybrid IgG1-IgG3 mAb was created, called IgGh47, which had the CH1-3 of IgG1 but a 47 amino acid IgG3 hinge. This hybrid IgG, exhibited IgG1s phenotype in binding both slightly greater opsonic ability than that of IgG3. This antibody was the only protective mAb in preventing bacterial dissemination in a subcutaneous challenge model in mice- the sum of the parts is greater than the whole. **D** The potent phenotype of IgGh47 over IgG1 was transferable to other GAS strains belonging to clinical isolates expressing different emmtypes. Similarly, in **E**, IgGh47 potently increased ADCP for 3 unique anti-spike mAbs. Created using biorender.

As discussed in **Chapter 5**, severe GAS infections, such as NSTI, have a high mortality rate despite the adequate use of antibiotics as part of standard care (SOC). Adding monoclonal antibodies to the existing SOC could potentially reduce mortality. Bahnan et al[170]. developed a broadly M protein-binding antibody with high affinity and strong opsonophagocytic function. This antibody significantly outperformed IVIG at just 1/40th of the dose (10 mg vs 0.4 mg) in protecting animals from systemic disease in an intraperitoneal infection model. However, this

antibody, called Ab25, was developed using the IgG1 backbone, and it was not clear whether this was the most potent opsonic subclass for this clone.

In Paper II, we generated all four IgG subclasses of this protective antibody and made several interesting observations. Starting with binding, we found that, as in Paper I, altering the constant domain of the antibody influenced the ability of the variable domain to bind to the M protein. While the IgG2 and IgG4 mAbs exhibited similar affinities to Ab25 IgG1, the IgG3 version showed a 13-fold lower affinity than IgG1 (28.3 nM vs. 2.2 nM). Surprisingly, however, the IgG3 mAb vastly outperformed IgG1 in opsonic ability when tested using THP-1 cells and primary neutrophils and monocytes. To understand the discrepancy between binding and function, the authors hypothesized that the increased hinge length of IgG3 could explain its enhanced efficacy despite its lower binding affinity. However, little research had been done to quantify the "improved flexibility" associated with a longer hinge (as discussed in Chapter 2). Therefore, we quantified the flexibility of IgG1 and IgG3 in relation to the Fc domain and its interaction with the M protein using molecular dynamics simulations. These simulations revealed a significant difference in how the Fc domain of IgG3 moves in 3D space compared to IgG1, occupying a much larger volume. This increased flexibility was theorized to facilitate a higher likelihood of interaction with Fc receptors (FcRs) and promote Fc-receptor clustering, which is important for immune activation and function. Additionally, the simulations showed that the interaction between IgG3 and M1 protein differed at the residue level from the interactions formed between IgG1 and M1. Different networks of hydrogen bonds and salt-bridges were formed by the two Fab domains that bind the M protein, suggesting that altering the constant domain does indeed influence antigen-binding properties, supporting the experimental data.

To test the hypothesis that increased 3D flexibility is crucial for phagocytosis, we generated hinge-modified versions of Ab25 IgG1 by replacing the 15-aa IgG1 hinge with IgG3 hinges of varying lengths (17, 32, 47, or 62 aa). We called these constructs IgGh17, IgGh32, IgGh47, and IgGh62. No differences in binding were observed for these constructs, suggesting that the hinge domain was not responsible for the decrease in antigen-binding ability of IgG3. However, increasing the hinge length correlated with improved opsonic ability, but only up to 47 amino acids, with the 62-aa version being inferior to the slightly shorter IgGh47. Notably, the IgGh47 version proved to be even more effective than the original IgG3 mAb.

In a subcutaneous infection model with mice, only the IgGh47 version of Ab25 protected against bacterial dissemination compared to the negative control, indicating that natural subclasses were less effective at clearing the infection than

this artificial IgG1-IgG3 hybrid version. The generalizability of this antibody backbone was further demonstrated by showing that its phenotype was conserved across multiple clinical isolates of GAS from diverse emm types.Finally, similar findings of IgGh47 superiority over IgG1 were observed in a SARS-CoV-2 anti-spike mAb setting. The RBD, NTD, and S2-binding mAbs (Ab11, 36, and 77) showed excellent opsonic function when expressed in this subclass. Ab36 was shown to be even more potent than DuomAb IgG3, the most potent opsonic cocktail consisting of IgG3 Ab94 and Ab81 from Paper I, which is protective against lethal Wuhan infection.

Thus, Paper II highlights not only a promising strategy for engineering a monoclonal antibody against *Streptococcus pyogenes* but also for targeting SARS-CoV-2 and possibly other pathogens. Hinge-engineering emerged as a promising approach, with antibody flexibility in 3D space playing a key role in efficient opsonic function. Furthermore, the findings provide stronger support for the notion that the constant domain can modulate the antigen-binding properties of the variable domain.

PAPER III

Protective Non-neutralizing anti–N-terminal Domain mAb Maintains Fc-mediated Function against SARS-COV-2 Variants up to BA.2.86-JN.1 with Superfluous In Vivo Protection against JN.1 Due to Attenuated Virulence

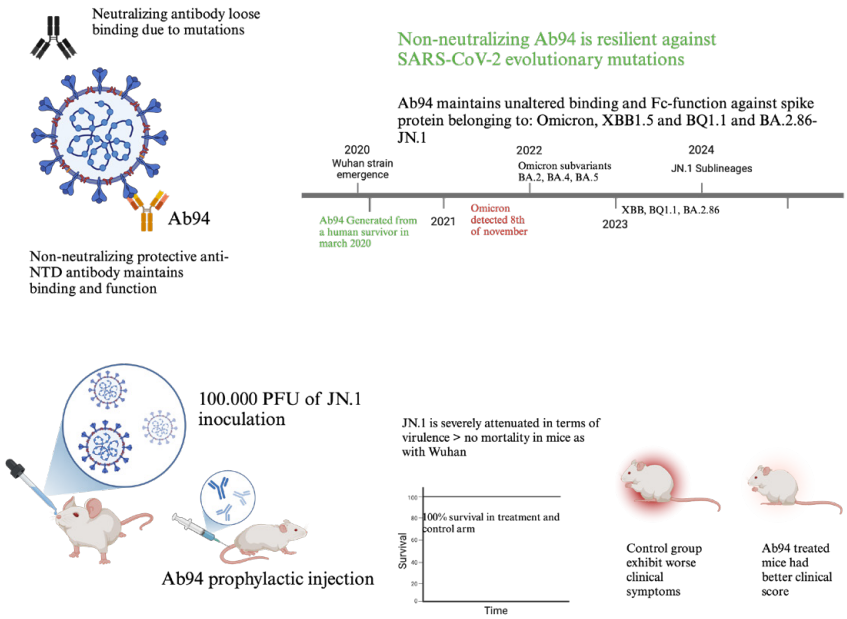

Figure 19. Overview of results from Paper III. A majority of neutralizing antibodies lost binding to the Omicron variant and its subsequent subvariants such as BA.2, BA.4, BA.5, XBB, BQ1.1, BA.2.86 and JN.1. Timeline is displayed to illustrate that Ab94 was generated against the WT strain in march 2020, while still maintaining its protective Fc-function against Omicron and the above mentioned subvariants. Unexpectedly, JN.1 has low disease manifestation, despite using what was a lethal dose of Wuhan inoculation (100.000 PFU, 100% mortality at day 5-6) in the K18-hACE2 murine model, where all mice had no loss of body weight or died. The control group experienced worse clinical symptoms compared to Ab94 treated mice, using a predetermined clinical scoring system. Created using biorender.

In paper I, we showed that non-neutralizing anti-NTD and anti-RBD mAbs, Ab94 and Ab81 respectively, protect against lethal Wuhan infection in K18-hACE2 mice[330]. However, with the emergence of Omicron and its subvariants, many neutralizing and non-neutralizing antibodies lost binding and therefore also Fab (neutralization) and Fc-function (as discussed in **Chapter 6**). Thus a key question arose: does the protective non-neutralizing antibody have any efficacy against the highly mutated antibodies?

Interestingly, we observed that all non-neutralizing antibodies used retained binding and Fc-function against the Omicron variant and subvariants such as BA.2, BA.4 and BA.5[347]. Furthermore, Omicron further mutated into XBB and the BQ1.1 variant at the end of 2022. Ab81 and Ab94 had both great opsonic function and binding to these variants. When encountering the even more heavily mutated BA.2.86 variant (relevant in end of 2023), Ab81 lost binding and function, while the NTD targeting Ab94 did not. BA.2.86 was quickly replaced by an off-shot variant called JN.1 which had one more mutation than BA.2.86 in the RBD, which enabled it to be more infectious, gaining dominance world-wide[320]. When assessing Ab94 against this variant in K18-hACE2 murine model at, what was a lethal dose of Wuhan virus (100.000 PFU), surprising results were observed. In terms of virulence, JN.1 was severely attenuated compared to Wuhan in the same animal model- no loss of mice or body weight was observed over the 10 day experiment. Viral titers in the lower respiratory tract were also low, even in the control group. However, when using a standardized clinical score system (based on symptoms), it became more clear that the Ab94 treated mice fared better than the negative control, suggesting at least some benefit to Fc-functions. This experiment was the first reported *in vivo* determinant of JN.1, which became an important variant throughout 2024 and its sub variants are circulating as of now in 2025 march. Although the benefit of monoclonal antibody treatment is difficult to assess now that the virulence of the virus has become significantly attenuated compared to Wuhan, the results from this study show that protective non-neutralizing antibodies, particularly against the NTD, retain strong binding and Fc-function to a greater degree than neutralizing ones- making them a potentially promising therapeutic strategy against mutated SARS-CoV-2 variants, and potentially emerging Betacoronaviruses.

PAPER IV

Hinge-engineered IgGh47 monoclonal antibodies exhibit ultrapotent Fc-mediated phagocytosis against SARS-CoV-2 and Betacoronaviruses through an unprecedented high-affinity binding to CD32 and CD16

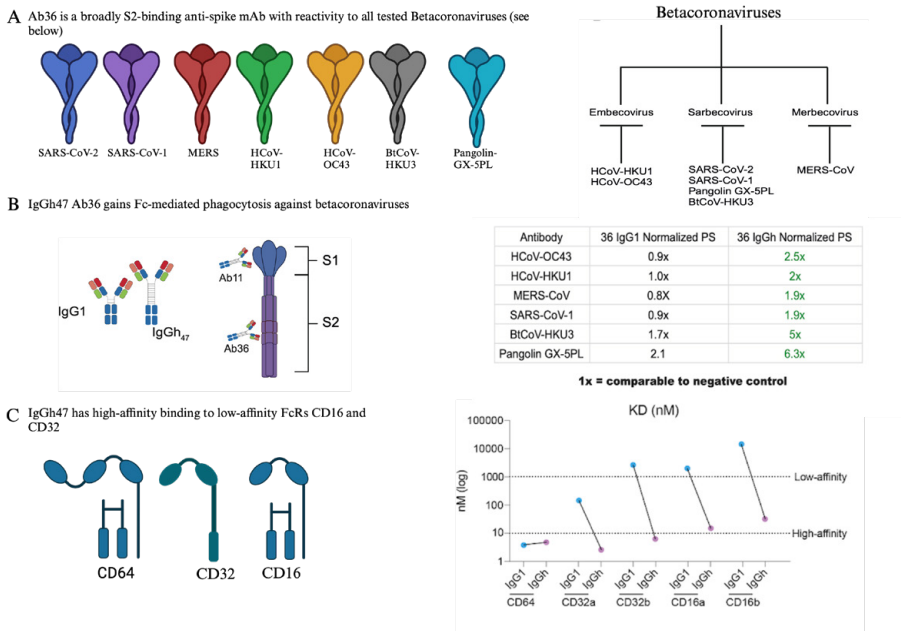

Figure 21. Overview of results in Paper IV. A Ab36 binds to all tested betacoronaviruses , spanning different subgenres within the family. **B** Ab36 IgG1 does not elicit Fc-functions against most tested spike protein. For the few it does there is a moderate increase compared to control. Engineering Ab36 into the IgGh47 variant results in enhanced Fc-function. **C** FcR affinity is notably increased for the traditionally classified low-affinity IgG receptors CD16 and CD32. The graph shows KD values for Ab11 IgG1 vs IgGh47, but similar trends were observed for Ab36.

Building on the previous work in this thesis (Papers I–III), we aimed to generate a potent opsonic, non-neutralizing antibody with cross-reactivity against multiple Betacoronaviruses that cause severe disease, such as MERS and SARS[87]. Our goal was to develop an opsonizing monoclonal antibody (mAb) with potentially protective non-neutralizing functions, serving as a proof of concept for a pan-Betacoronavirus treatment to enhance pandemic preparedness and combat emerging SARS-CoV-2 variants. We identified Ab36 as a broadly S2-binding mAb with specificity against multiple Betacoronaviruses, including HCoV-HKU1, HCoV-OC43, BtCov-HKU3, Pangolin-GX-5PL, MERS, and SARS-CoV-1. These spike proteins originate from diverse Betacoronaviruses that infect various species,

including bats, pangolins, and humans. Engineering the original IgG1 and IgG3 into the IgGh47 subclass produced a potent opsonic variant[348]. Ab36 IgGh47 demonstrated significantly enhanced ADCP activity against multiple Betacoronaviruses compared to its IgG1 counterpart, which elicited little to no Fc-mediated response for most spike proteins. A similar effect was observed for the Ab11 clone. Notably, the enhanced ADCP activity extended to the mutated SARS-CoV-2 variant BA.2.86, suggesting that these antibodies target conserved epitopes on the spike protein. To investigate the mechanism underlying this improved ADCP, we analyzed Fc receptor (FcR) affinity for the IgG1 and IgGh47 versions of Ab11 and Ab36 using surface plasmon resonance (SPR). Interestingly, both IgGh47 variants exhibited strong nanomolar-range binding to the low-affinity FcRs CD16a/b and CD32a/b an effect not previously reported with hinge-engineered modifications[195,208–211]. This work highlights an effective strategy for antibody development, where candidates are first selected based on epitope targeting and subsequently optimized through hinge engineering to enhance FcR affinity, resulting in a potent opsonic mAb. Hinge-engineering into IgGh47 potentially has broader applications in other therapeutic areas given the FcR affinity increase observed.

DISCUSSION AND FUTURE PERSPECTIVE

Monoclonal antibody therapeutics represent the fastest-growing form of medicine, with an estimated market value in the hundreds of billions of dollars[349,350]. In clinical practice, these therapies have been widely utilized for indications in rheumatology, oncology, and infectious diseases [349,350]. The desired effects of monoclonal antibodies vary depending on their application. For instance, in some cases, Fab-mediated effects are critical, such as neutralization of a viral virulence factor, whereas in other scenarios, antibody-dependent cellular cytotoxicity (ADCC) targeting tumors is preferable[32]. Accordingly, antibody engineering strategies must be tailored to specific indications. Through careful clinical and preclinical research, we can identify which functions are most beneficial *in vivo.* For example, Rituximab depletes CD20+ cells through complement-dependent lysis and ADCC[181,182]. Engineering strategies such as afucosylation, GASDALIE mutations, or hexamer-promoting mutations to enhance C1q activation can amplify these effects. Advances in antibody engineering now enable the customization of already highly specific antibody drugs for potentially greater therapeutic efficacy (once the mechanism of action is thoroughly understood).

CHALLENGES IN DEVELOPING ENGINEERED MONOCLONAL ANTIBODIES

Despite these advancements, generating engineered monoclonal antibodies (mAbs) with clinical efficacy remains challenging. Preclinical work must involve relevant animal models and disease manifestations that are transferable to human settings. Furthermore, robust but clinically relevant assays are essential for screening antibody candidates prior to advancing to clinical trials. For SARS-CoV-2 research, the K18-hACE2 murine model has proven reliable for mimicking severe COVID-19 disease[343]. Live-virus neutralization assays (and to some extent pseudovirus assays) have demonstrated correlation with protection observed in clinical trials for vaccines and monoclonal antibody candidates[309,351]. However, measuring Fc-mediated functions is more complex than assessing Fab-mediated neutralization, where the readout is straightforward (e.g., reduced infection or activity). Using Fc-mediated phagocytosis of SARS-CoV-2 as an example: how could we determine whether the PAN-method or any other ADCP method correlates with *in vivo* function in humans? Ideally, antibody candidates should be screened using multiple complementary assays that assess various modalities. This approach was pursued in Paper I[330], where Fc-mediated phagocytosis was measured using streptavidin-conjugated spike protein beads as a model for virions. By employing flow cytometry (FACS) and microscopy to measure the internalization rate of human neutrophils,

we achieved consistent results across methods. However, while utilizing human neutrophils brings the assay closer to an in vivo setting, spike-protein microsphere beads differ significantly from real virions. Virions possess membranes with distinct properties such as density, weight, and diameter compared to microsphere beads. Moreover, translating *in vitro* and *ex vivo* experiments into efficacy within animal models like the K18-hACE2 murine model presents additional challenges. The murine immune system differs substantially from humans in terms of phagocyte Fc receptor (FcR) expression and types of FcRs present despite structural and functional similarities[352] .This issue can been partially addressed by transferring human peripheral blood mononuclear cells (PBMCs) into irradiated mice or using humanized FcR mice (mice expressing human FcRs on murine phagocytes)[162,345]. More detailed studies can be conducted by knocking out specific humanized receptors to examine individual receptor contributions. However, such reductionist approaches may overlook nuances and interactions between receptor activations. Furthermore, these animal models remain highly artificial, and their translational relevance is uncertain. Bridging the gap between *in vitro*, *ex vivo*, and *in vivo* experiments to human settings remains a significant challenge. This issue is crucial to consider when interpreting the translational relevance of results presented in this thesis.

Insights into Antibody Constant Domain Influence on Antigen-Binding Properties

As discussed in **Chapter 2**, it has long been believed that the constant domain of an antibody does not influence the binding properties of its variable domain. However, independent research groups have demonstrated that altering the subclass or class of an antibody can indeed significantly impact antigen binding[100]. For SARS-CoV-2, this effect can be particularly dramatic, with a gain of function in terms of binding and neutralization when shifting from IgG1 to IgG3 or IgA[104,198,199], as seen with therapeutically approved monoclonal antibodies. This thesis builds upon these findings.

In Papers I and II[330,348], we observed that modifying the constant domain could either enhance or reduce binding affinity by several-fold. For instance, Ab25 IgG3 exhibited a 13-fold reduction in affinity compared to its IgG1 counterpart. Similarly, Ab57 and Ab11 showed affinity changes ranging from 10 to 100-fold, depending on the assay used in SARS-CoV-2 studies. Interestingly, Ab57 displayed higher affinity when expressed as IgG3, while Ab11 showed greater affinity as IgG1, despite their native subclasses being IgG1 and IgG3, respectively, in the human

donor. These results suggest that the native subclass expressed by a B-cell receptor (BCR) may not always be optimal for antigen affinity. Modifying the constant domain could enhance BCR diversity alongside somatic hypermutation (SHM) and VDJ recombination processes.

Molecular dynamics (MD) simulations conducted on Ab25 IgG1 and IgG3 (Paper II) provide potential explanations for these experimental findings. Altering the constant domain modifies molecular interactions between the epitope and paratope by forming altered hydrogen bonds and salt bridges. These hypotheses could be tested experimentally by introducing mutations into key residues within the variable domain to observe their impact on antigen binding. Additionally, structural techniques such as crystallography or cryo-electron microscopy (Cryo-EM) could further elucidate these interactions at a molecular level. While MD simulations offer valuable insights into molecular mechanisms, it is important to recognize their limitations as models rather than direct representations of reality.

Exploring how constant domain switching influences antibody-antigen interactions remains an intriguing avenue for future research and is gaining traction, as evidenced by the increasing number of reviews on this topic. This thesis provides evidence that challenges the traditional dogma, which holds that variable domains function independently from constant domains.

Importance of non-neutralizing antibody function against disease-causing Betacoronaviruses

At the beginning of the COVID-19 pandemic, much focus was placed on neutralizing functions to provide immunity against SARS-CoV-2. However, evidence quickly emerged showing that non-neutralizing functions were also important for protection, especially after the emergence of the Omicron variant, when neutralizing titers significantly decreased as discussed in **Chapter 6.** This thesis contributes to other observations, including Paper I, which presented the first instance of a non-neutralizing RBD monoclonal antibody (Ab81) protecting against lethal infection (100,000 PFU) of the virulent Wuhan strain[330]. Similarly, the observation that Ab94 (a non-neutralizing NTD mAb) was protective in terms of overall mortality in the same experiment confirmed previous findings where this mAb prevented weight loss, similar to a neutralizing antibody. Paper I also demonstrated how these protective non-neutralizing functions could be enhanced by using IgG3 cocktails, while the IgG1 versions exhibited only modest opsonic potential. However, the potent opsonic effect did not translate into increased survival in the K18-hACE2 murine model, which could be due to several factors.

First, the animal model does not have human phagocytes that express human Fc receptors, making it difficult to compare across species. Additionally, no experiments were conducted with murine phagocytes to allow for easier interpretation. Second, as previously discussed, the translational relevance of various assays in human or murine models has not been fully established, and promising *in vitro* and *ex vivo* results should be interpreted cautiously.

Nevertheless, monoclonal antibody development against SARS-CoV-2, and Betacoronaviruses in general, would likely benefit from Fc-engineering strategies to improve ADCP and ADCC. It is important to note that aberrant glycosylation patterns, which result in excessive ADCC and alternative complement activation, have been linked to severe COVID-19 in patients (which is discussed in this review[347]). This raises concerns about the proinflammatory effects of mAb therapeutics.

There are also developmental aspects to consider. For instance, the IgG3 subclass is known to have issues with aggregation, weaker thermal stability, and potentially greater susceptibility to hinge cleavage by proteases, which could affect its half-life[97]. As discussed in **Chapter 2**, these issues can be addressed through point mutations, but it is not guaranteed that these mutations, either individually or together, will not affect the Fc functionality of the highly engineered IgG3 mutant. Interestingly, the hybrid IgGh47 seems to bypass many of these issues by only incorporating the hinge of IgG3. As shown in Papers II and IV, this hybrid subclass dramatically increased ADCP. Furthermore, Fc receptor affinity for various low-affinity receptors (including the inhibitory CD32b) was significantly enhanced, suggesting that ADCC might also be improved with this subclass, although this was not explored further in this thesis. The hybrid IgGh47 exhibits a half-life comparable to IgG1 and higher than IgG3, as demonstrated by FcRn receptor affinity and serum concentration in the animal experiments in Paper II. However, aggregation assays and thermal stability analyses need to be conducted to better understand the feasibility of this subclass, along with toxicology studies.

Nevertheless, based on the work presented in Papers I-IV, using either IgG3 or IgGh47 appears to be a promising alternative to the widely used IgG1 subclass for developing both non-neutralizing and neutralizing monoclonal antibodies targeting SARS-CoV-2 and other disease-causing Betacoronaviruses. These subclasses could harness potent opsonic abilities for protective therapeutic efficacy in patients which are not immunocompromised in terms of lacking phagocytes and NK-cells.

A NEW TREATMENT STRATEGY FOR INVASIVE *STREPTOCOCCUS PYOGENES* INFECTIONS?

As outlined in the introduction, the central aim of this thesis is to explore how engineered monoclonal antibodies (mAbs) can improve opsonophagocytic function, specifically targeting *Streptococcus pyogenes* and other infectious pathogens. In this section, we discuss the potential of Ab25 as a promising therapeutic candidate and how its superior opsonophagocytic activity aligns with our research objectives.

Severe streptococcal diseases, particularly STSS (streptococcal toxic shock syndrome) and NSTI (necrotizing soft tissue infections), present significant challenges in treatment today as discussed in **Chapter 5**[247]. Mortality rates can reach up to 60%, and those who survive often experience high morbidity, with amputations being unfortunately common (20%). Furthermore, young, healthy (non-immunocompromised) patients with long life expectancies are also affected. This creates an incentive, both from a healthcare economics and moral perspective, to pursue research that could lead to increased survival and reduced morbidity.

Currently, the standard treatment consists of penicillin and adjunctive clindamycin. However, as noted, these treatments have limited success in reducing mortality, with high-dose intravenous immunoglobulin (IVIG) often being administered as a complementary therapy[250]. There is an alarming increase In clindamycin resistance, more than 300% increase according to CDC in the US during the year 2011-2018[251,353,354] . While IVIG has *ex vivo* demonstrated toxin inhibition, its clinical impact is often negligible, likely due to weak opsonic activity and a lack of specificity[249,250]. This is where Ab25, with its enhanced opsonophagocytic activity[170], shows potential to significantly outperform IVIG in promoting bacterial clearance, directly addressing the current treatment limitations in addition in an era where clindamycin resistance is growing.

As discussed in **Chapter 5**, the M protein is a key candidate for vaccine development because it triggers host humoral immunity, generating opsonic antibodies targeting both its hypervariable region and its conserved domain closer to the bacterial surface. However, the hypervariable region is highly strain-specific, and the M protein can inhibit phagocytosis by opsonic antibodies against the conserved domain through binding with albumin and fibrinogen[269]. Consequently, antibodies in IVIG that specifically target the M protein might not recognize the infecting strain. Even if they do, their action could be inhibited by the binding of host proteins. Additionally, the activities of EndoS and IdeS enzymes have been shown to reduce the opsonic effects of IVIG. A recent study by Toledo and Bratanis et al.[267] demonstrated that mice treated with high-dose IVIG did not experience

reduced bacterial load when these enzymes were active. However, when IVIG was not inhibited by IdeS and EndoS (via IP inoculation instead of subcutaneous infection), IVIG did reduce bacterial colonization in various organs.

Today's standard treatment faces several challenges, primarily due to insufficient bacterial reduction leading to too high toxin production which damages the host . Unlike IVIG, which is a polyclonal antibody mixture with a broad spectrum but limited opsonic activity, Ab25 is a monoclonal antibody engineered specifically to enhance opsonophagocytosis. This focus allows Ab25 to achieve much greater efficacy per dose, as demonstrated in both subcutaneous and intraperitoneal challenge models, where it outperformed IVIG by a significant margin (at 2.5% of the dose). This highlights how engineered mAbs, specifically tailored to target bacterial antigens, hold promise for more effective treatments than current polyclonal therapies. Additionally, Ab25 offers broad-strain coverage, including major disease-causing strains such as M1, M5, M12, and M28, and does not cross-react with human tissues, thus eliminating concerns about autoreactive antibodies due to molecular mimicry.

These findings demonstrate that Ab25 significantly outperforms current adjunctive treatment options like IVIG in reducing bacterial load, supporting our hypothesis that engineered monoclonal antibodies with enhanced opsonophagocytic potential can address the limitations of traditional treatments. This result aligns with the key aim of this thesis to evaluate the therapeutic potential of monoclonal antibodies in severe bacterial infections. Furthermore, Ab25 is currently the only human monoclonal antibody developed against the M protein. Its distinct dual-Fab/bispecific binding patterns, may be a key factor contributing to its favorable cross-strain reactivity and enhanced opsonic function. As a result, the likelihood of generating a comparable antibody candidate remains to be determined but could possibly experience a similar binding phenotype. However, previous work on Ab25, combined with the findings in this thesis, improves our understanding of how to generate a potent opsonic anti-streptococcal monoclonal antibody.

While these findings have potential applications in other severe bacterial diseases, it is important to emphasize that each antigen-antibody interaction is unique. Therefore, the strategies used to engineer Ab25 should be carefully considered for each new pathogen, highlighting the need for tailored monoclonal antibody designs, as explored throughout this thesis. This focus ensures that our approach can be successfully applied to *Streptococcus pyogenes* while providing a framework for future research in other bacterial infections.

This thesis builds upon the promising antibody, with the IgGh47 version showing particularly strong protective effects *in vivo.* The protective efficacy of this subclass is attributed to its ability to promote phagocytosis of bacteria, surpassing the activity of both natural IgG1 and IgG3 subclasses of Ab25. Ab25 IgGh47 represents a promising candidate for further exploration in preclinical animal studies, as well to assess development ability aspects as discussed in previous sections. This thesis contributes to understanding how we might enhance the protective effects of monoclonal antibodies against *Streptococcus pyogenes*, while inadvertently identifying a promising candidate for future therapeutic use against severe disease. However, much more work is needed to translate these promising *in vitro* and *in vivo* findings from academic settings into tangible clinical outcomes that could theoretically save patients' lives, reduce risk of amputation and reduce the impact severe infection has on organ systems after hospital discharge.

CONCLUSION

This thesis investigated how modifications to the constant domain of monoclonal antibodies can influence their binding properties and Fc-mediated phagocytosis. It was demonstrated that the constant domain can modulate antigen binding in two independent biological systems, contributing to a growing body of literature that challenges the traditional view of antibody independence between the variable and constant domains (Paper I-II)[330,348]. This work provided evidence that non-neutralizing Fc-functions not only provide protection against the original SARS-CoV-2 strain but also offer protection against highly mutated variants such as JN.1 (Paper III)[347]. These non-neutralizing antibodies, which retain their function against mutated strains for more than four years, represent a promising tool for combating future pandemics caused by beta-coronaviruses, where traditional neutralizing antibodies often lose efficacy (Paper III-IV). In the context of streptococcal disease, this thesis extends previous findings by demonstrating that monoclonal antibodies targeting the M protein show promise as novel therapeutics (Paper II). The engineered IgGh47 subclass, with its enhanced FcR affinity compared to the parent IgG1 and IgG3 (Paper IV), proves to be a potent tool for improving opsonic function, thus expanding the therapeutic potential of monoclonal antibodies in this area. In conclusion, this thesis underscores the therapeutic potential of Fc-engineered monoclonal antibodies in enhancing opsonization, with significant applications for both SARS-CoV-2 and *Streptococcus pyogenes*. By improving FcR interactions through subclass engineering, these antibodies hold promise for advancing therapeutic strategies in the fight against these and potentially other

infectious pathogens. Thus, Fc-engineering emerges as a crucial tool in the design of next-generation monoclonal antibody therapies.

The research executed in Paper I-IV reinforces the thesis title, *"**Fc-Engineered Antibodies Against SARS-CoV-2 and Streptococcus pyogenes: Therapeutic Potential via Enhanced Opsonization***" by demonstrating how Fc-engineering can enhance the efficacy of monoclonal antibodies in potentially combating these critical pathogens with clinical application.

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The Lab family
The aunts and uncles

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The siblings

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APPENDIX (PAPERS I-IV)