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2019

Document Version:
Publisher's PDF, also known as Version of record

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LUND
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DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at the Biomedical Centre, Belfragesalen (BMC D15) on the 25th of October 2019 at 09.00.

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DOCTORAL DISSERTATION

Date of issue 25-10-2019

Sponsoring organization

Title and subtitle
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Abstract

Antibodies are an essential part of the human immune system, and antibody mediated immunity has been an area of interest for many researchers for almost a century. An accumulation of knowledge regarding antibody structure, glycosylation and receptor interactions has contributed to the current understanding of antibody mediated immunity. It has more recently become evident how bacteria and other microorganisms evade host recognition and eradication through specific antibody degradation or modification. The importance of antibody glycosylation and how glycan modification can fine-tune the elicited immune response has also contributed to the development of antibody-based drugs with improved clinical efficacy. In turn these insights have paved the way and created a need for the development of biotechnological methods and tools to specifically engineer antibodies with defined properties, for analysis to ensure quality and safety, and for improved antibody purification.

This thesis highlights the importance of glycosylation for antibody function and presents different aspects and applications of antibody modifications by bacteria. We show, for the first time, activity of the IgG-specific Streptococcal endoglycosidase EndoS during *Streptococcus pyogenes* infection, clearly demonstrating that EndoS contributes to *S. pyogenes* pathogenesis and bacterial survival in the context of adaptive immunity. Further this thesis presents the use of bacterial enzymes as antibody modifying tools and their potential as binding reagents for selective antibody purification. The identification and characterization of two novel proteases, BspK and BspE exhibiting unique IgG and IgA cleavage profiles respectively, from *Bdellovibrio bacteriovorus* highlights the potential of using *Bdellovibrio* as a source for the identification of novel enzymes with biotechnological applications. Finally, I present the development of a novel method for selective antibody purification, using the inactive variants of the bacterial enzymes EndoS and EndoS2, ensuring the purification of native, correctly folded and modified antibodies.

Key words: Immunoglobulins, immunomodulation, antibody mediated immunity, glycosylation, bacterial virulence, bacterial enzymes, biotechnology, *Streptococcus pyogones, Bdellovibrio bacteriovorus*

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-7619-829-2
Recipient's notes	Number of pages 87	Price:
	Security classification	

Signature  Date 2019-09-19

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ISBN 978-91-7619-829-2
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity.

- Albert Einstein

Table of content

Abstract

Antibodies are an essential part of the human immune system, and antibody mediated immunity has been an area of interest for many researchers for almost a century. An accumulation of knowledge regarding antibody structure, glycosylation and receptor interactions has contributed to the current understanding of antibody mediated immunity. It has more recently become evident how bacteria and other microorganisms evade host recognition and eradication through specific antibody degradation or modification. The importance of antibody glycosylation and how glycan modification can fine-tune the elicited immune response has also contributed to the development of antibody-based drugs with improved clinical efficacy. In turn these insights have paved the way and created a need for the development of biotechnological methods and tools to specifically engineer antibodies with defined properties, for analysis to ensure quality and safety, and for improved antibody purification.

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Original papers

I. Eleni Bratanis, Henrik Molina, Andreas Naegeli, Mattias Collin, Rolf Lood. BspK, a serine protease from the predatory bacterium *Bdellovibrio bacteriovorus* with utility for analysis of therapeutic antibodies. *Applied and Environmental Microbiology* 2017 Feb 15; 83(4)

II. Eleni Bratanis, Rolf Lood. A novel broad-spectrum elastase-like serine protease from the predatory bacterium *Bdellovibrio bacteriovorus* facilitates elucidation of site-specific IgA glycosylation pattern. *Frontiers in Microbiology* 2019 May 03; 10 971

III. Andreas Naegeli, Eleni Bratanis, Christofer Karlsson, Oonagh Shannon, Raja Kalluru, Adam Linder, Johan Malmström, Mattias Collin. *Streptococcus pyogenes* evades adaptive immunity through specific IgG glycan hydrolysis. *Journal of Experimental Medicine* 2019 Jul 01; 216(7) 1615–1629

IV. Eleni Bratanis, Maria Allhorn, Oskar Lundin, Andreas Naegeli, Mattias Collin. Selective purification of native human IgG from complex samples using bioengineered EndoS. *Manuscript* 2019

Introduction

The co-evolution of bacteria with the human host has led to the development of strategies and mechanisms to modulate and neutralize the host immune system in order to persist. Such mechanisms include the production of bacterial enzymes with the capacity to neutralize antibody mediated immune responses, which has significant effects on the capacity of the host to respond to and counteract the invading bacteria. During the last decades it has become increasingly evident that modulation of antibody glycosylation fine-tunes the immune response, and several associations have been shown between general shifts in antibody Fc-glycosylation profiles and diseases including reumatoid arthritis and systemic lupus erythematosus. Further research and a better understanding of the bacterial mechanisms of antibody modulation has resulted in the application of bacterial enzymes as novel biotechnological tools and potential therapeutic agents.

The aim of this thesis is to give an overview of the function, and importance of bacterial antibody modulation during infection, and to illustrate how bacterial strategies are currently being utilized to develop novel biotechnological tools and therapeutic agents.

1. Antibody mediated immunity

The immune system is best described as an intricate, dynamic and interactive network including many different players and compartments, designed to protect the host from invading microorganisms and other foreign antigens. The immune system is commonly divided into innate and adaptive responses, based on the speed and specificity of the response. However, in practice these different branches are finely intertwined with continuous interaction. The innate immune response is often described as a rapid and non-specific response, identifying and acting on general threats, often leading to damage of host tissues. On the other hand, the adaptive immune response is acquired, with finely tuned antigen-specific reactions based on immunological memory, involving a tightly regulated and finely orchestrated interplay between different immune cells.

Interestingly, bacteria are an integral part of human biology, involved in numerous essential functions of normal host physiology including nutrient metabolism, antimicrobial protection and immunomodulation [1–3]. This symbiosis between the human and bacterial cells relies on the confinement of the bacteria to the correct localization within the body, and the integrity of the barriers which keep them there. The skin is the first line of defense and together with the mucosal membranes constitutes an important barrier against infection. If the skin is breeched by a cut, a commensal bacterium entering the blood could cause a severe infection. However, a bacterium entering the blood does not necessarily need to result in a major infection with poor outcome. If the outer barriers are breeched the immune system, comprised of specialized immune cells, organs, signal substances and molecules is designed to neutralize the invading threat and protect the host. Bacteria have co-evolved with humans and developed mechanisms to evade the immune responses (this will be described in more detail in later chapters). The immune system has the ability to recognize self from non-self, and to distinguish between different invading bacteria or other microorganism, adjusting and tuning the response accordingly. Invading microorganisms are initially recognized by pattern recognition receptors, including the array of different toll-like receptors, that recognize distinct microbial components known as pathogen associated molecular patterns (PAMPs) to immediately activate immune cells including macrophages and dendritic cells [4,5]. Bacterial PAMPs are generally essential bacterial components including cell wall constituents like lipopolysaccharide (LPS), peptidoglycan or bacterial genomic

DNA [4]. The recognition of microorganisms or infected cells by circulating antibodies accounts for another essential branch of the immune system, which through binding to their target epitopes have the ability to neutralize and clear the invading bacterium through phagocytosis or direct killing [6].

Antibody structure and diversity

Antibodies, or immunoglobulins, are the major products of the adaptive immune system, serving both as receptors and as effector molecules. Beyond their role in neutralization and clearance, antibodies also mediate effector functions through the interactions with different receptors or the complement system. By targeting the invading microorganism or other harmful substance specifically, by recognizing unique epitopes, antibodies enable the immune system to respond with precision without damaging self. This requires pre-exposure to the pathogen or antigen, which primes the antibody producing B-cells to generate specific antibodies, enabling the immune system to mount a rapid and targeted response upon reinfection with the same infectious agent. This is the basis for vaccination, and results in a broad repertoire of circulating antibodies and memory cells, with the capacity to quickly start producing specific antibodies upon activation, which depends on our individual history of infections.

The effectiveness of the antibody mediated immune response lies in the combination of the individual antibody specificity, together with a vast antibody diversity and repertoire comprised of approximately 10^9 -10^{12} unique antibodies [5,7]. The production and selection of antibodies by B-cells is an intricate, strictly regulated process ensuring antigen specificity and self-tolerance. Antibody mediated autoimmune diseases including reumatoid arthritis (RA) and systemic lupus erythematosus (SLE), reflect faulty tolerance mechanisms, allowing maturation of auto-antibody (antibodies that target and react with self proteins, cells and tissues) producing B-cells and their differentiation into antibody producing plasma cells [8,9]. The flaws in central and/ or peripheral tolerance mechanisms results in the production of circulating auto-antibodies that react too strongly, and/ or towards inappropriate epitopes. Robust epidemiological data indicates a parallel increase in the prevalence of both allergies and autoimmune diseases, a trend that is particularly evident in the industrialized countries [10,11]. The underlying mechanisms and causalities are still a subject of investigation and debate, however, the correlation with a decrease in the incidence of major infectious and parasitic diseases is being proposed by some as a relevant factor. How these pathogenic responses can be modified through the action of bacterial enzymes will be discussed in more detail in chapter 3. This chapter gives a general overview of the antibody structure with a focus on IgG and IgA.

There are five distinct human antibody isotypes IgA, IgD, IgE, IgG and IgM, all sharing a general Ig-architecture comprised by four polypeptide chains, two class-specific (γ, α, μ, δ, ε) heavy chains (HC) each linked to a light chain (LC). The HC consist of an N-terminal variable domain (VH) and, depending on the antibody isotype, three (IgG, IgA, IgD) or four (IgE, IgM) constant domains (CH1-3/4). Similarly, the LC is composed by an N-terminal variable (VL) and a constant (CL) domain. The typical Y-shaped antibody structure is formed by the association of the two LCs with the two HCs through a flexible hinge region, arranging the antibody into two distinct functional units comprised by the Fab-regions (antigen-binding fragment), and the Fc-region (fragment crystallizable) responsible for mediating responses including antibody dependent cell mediated cytotoxicity (ADCC), antibody dependent cell mediated phagocytosis (ADCP) or complement dependent cytotoxicity (CDC) (Fig. 1) [12,13]. The different antibody isotypes display distinct structural features that affect antibody function. For example, IgM mainly forms pentamers displaying enhanced avidity to antigens and the ability to bind and activate complement. IgE in turn, is traditionally recognized as a mediator of allergic reactions and immune responses against helminths. More recently, the extremely high affinity between IgE and its receptor (FcεR) (10^{10} M^{-1}) has rendered IgE an interesting candidate to develop for therapeutic applications. Compared to many of the other antibody isotypes the lack of a hinge region confers an increased structural rigidity to IgE. Structural studies however have shown that the IgE-Fc has the ability to undergo extreme conformational changes, showing an alternative flexibility independent of the hinge region [6,14].

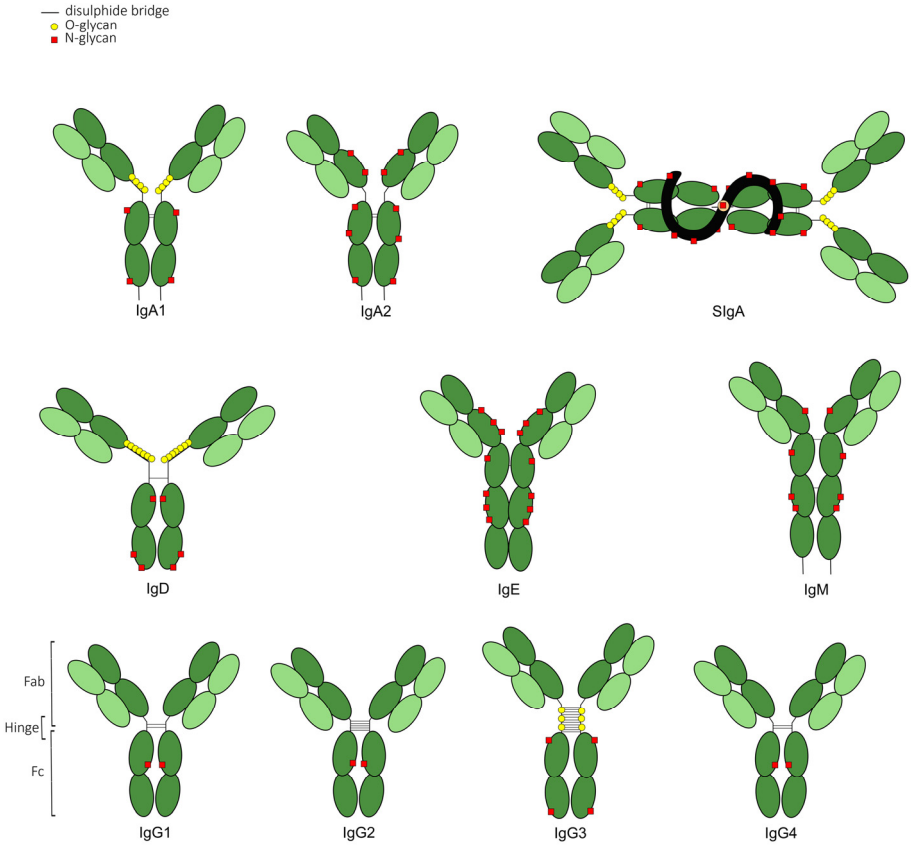

Fig. 1

Schematic figure showing the basic structure of the different human antibody isotypes and subclasses (IgA1-2 and SIgA, IgD, IgE, IgM (the IgM pentamer is not included in the figure) and IgG 1-4. Human antibodies consist of two functional domains; the antigen-binding fragment (Fab) domain that binds to antigens; and the crystallizable fragment (Fc) domain that binds to host sensors that deploy effector functions linked by a hinge region. Each antibody molecule is comprised of four chains with two identical heavy chains (HC, dark green) and two identical light chains (LC, light green). The heavy- and light- chains are further divided into variable (VH or VL) domains and constant (CH or CL) domains. IgA and IgM exist both as monomers and/or multimers (mainly represented by IgM pentamers and IgA dimers, linked by disulphide bridges, polypeptide J chains. Importantly for IgA the secretory component associates with dimeric IgA during translocation across the mucosal epithelial layers to external secretions, forming secretory IgA (SIgA). Antibody flexibility and conformation is influenced by the length and flexibility of the hinge, the number and location of disulphide bridges as well as the number and location of O-linked and/ or N-linked glycosylation. The final antibody structure, -flexibility and -glycosylation impacts the interaction with immune receptors, and thereby affect the capacity of an individual antibody to elicit effector functions.

It is easily forgotten, but we are constantly surrounded by a multitude of different microbes, generating an even larger array of different antigens, which the immune system needs to recognize in order to correctly counteract the potential threat. The size and diversity of the antibody repertoire is fundamental in order to specifically target, and rapidly neutralize such a vast number of potential epitopes. Antibody diversification and the generation of highly specific antibodies requires B-cell activation, which leads to the differentiation from naïve cells that have not been exposed to an antigen into specialized antibody producing plasma cells or memory cells. This activation can proceed by two distinct routes that is either dependent or independent on interactions with helper T-cells (Th cells). T-dependent antigen activation (TD-Ag) occurs following engagement of the B-cell receptor (BCR), a transmembrane surface immunoglobulin receptor, with a specific membrane associated or soluble antigen. Following binding, B-cells can take up the antigen, process and present it in association with the major histocompatibility complex II (MHC-II) leading to the recruitment of $CD4^+$ T-cells. T-cell recognition of the MHC-II antigen complex on the B-cell through the T-cell receptor, together with interactions of co-stimulatory molecules B7 (B-cell) and CD28 (T-cell), activates the T-cell and leads to cytokine production and secretion. This is followed by expression of CD40L ligand on the Th-cell and interaction with the CD40 receptor on the B-cell, providing a second signal to activate the B-cell. This is followed by an upregulation of various cytokine receptors on the B-cells and binding of the cytokines secreted by the activated T-cells, leading to B-cell proliferation and differentiation. TD-Ag drives differentiation into either extrafollicular short-lived plasmablasts producing low-affinity antibodies, or long-lived plasma cells producing high-affinity antibodies, and memory B-cells that provide long-lasting protection from secondary challenges with the same antigen. B-cell activation is a prerequisite for entering the germinal centers, specific sites within secondary lymphoid organs including lymph nodes and the spleen, where the antibody diversification by class switch recombination and antibody affinity maturation takes place [15,16]. T-independent antigen (TI-Ag) activation can be achieved either through extensive cross-linking of the BCRs by highly repetitive bacterial cell wall structures e.g. capsular polysaccharides and flagellin, or via BCR and TLR co-stimulation. Whilst TD-Ag activation often results in antibody isotype switching and affinity maturation TI-Ag does not [15–17].

The primary mechanisms for generating a large antibody repertoire include the genetic V(D)J recombination, where the variable (V), diversity (D) and joining (J) genes are joined with various non-templated junctions, producing unique immunoglobulin HC and LC. B-cell activation by antigen stimulation results in the production of specific antibodies, that are further diversified through somatic hypermutation (SHM). SHM is typically confined to the immunoglobulin variable region, resulting in improved affinity for the target epitope, generating a high-

affinity antibody repertoire characteristic of a mature immune response. Secondary mechanisms of diversification contribute to further extend the heterogeneity of the antibody population, such mechanisms include V(DD)J recombination, affinity maturation and antigen contact by non-CDR regions, and SHM-associated insertions or deletions [18]. Antibodies are further diversified through post-translational modifications (PTMs) including N- and O-glycosylation, deamination and chain trimming.

IgG is one of the most abundant proteins in circulation (10-20% of plasma proteins), and by far the most abundant serum antibody (7-15 mg/ml). The human IgG pool is comprised of four structural subclasses, IgG1> IgG2 > IgG3 > IgG4 (in order of decreasing abundance). Although highly conserved on the amino acid level (90 % identical), the main differences are located in the hinge region and upper CH2 domains. This variability results in distinct subclass profiles displaying both immunochemical and functional differences, including properties such as half-life, placental transport, antigen binding, immune complex formation and the interactions with IgG Fc-receptors (FcγR) and the complement component C1q [12,19]. IgG variability is further increased by allotypic variations in the IgG HC, as well as differential glycosylation which drastically expands the possible IgG glycoforms [12,13,19,20] (Fig 2). The type of the antigen and the route by which it enters the body directs the immune response, including the production of the most suitable antibody isotype and subclass. It has been suggested that the different IgG subclasses, due to their distinct interactions with various receptors, display subclass-specific roles during a natural infection. IgG1 and IgG2 have been implicated as the primary IgG subclasses in the response to bacterial polysaccharide antigens, and IgG2 deficiencies have been associated with increased susceptibilities to certain bacterial infections. IgG3 is regarded as a potent pro-inflammatory antibody particularly effective in inducing effector functions, however due to its elongated hinge region and the exchange of a histidine for arginine at position 435, it has a shorter half-life compared to all other IgG subclasses [12,21–24]. IgG4 antibodies are, together with IgE, often produced in response to repeated or chronic exposure to antigens/ allergens, e.g. during immunotherapy through hyposensitization [12,20,25,26]. Importantly the subclass distribution and functions vary between species, a factor that should be considered in the context of animal models.

IgA is the main antibody isotype found in secretions and mucosal membranes, accounting for approximately 15% of total body immunoglobulins. IgA mediates protection against pathogens through neutralization, agglutination and clearance. More recently IgA has also been recognized for its ability to induce effector functions such as phagocytosis, ADCC and release of inflammatory mediators, through interactions with Fc alpha receptor I (FcRαI). In the circulation, IgA referred to as serum IgA, consists mainly of monomeric IgA1. Serum IgA exists as two main subclasses, IgA1 and IgA2, and two additional allotypes IgA2m(1) and

IgA2m(2). Structural differences between the subclasses include an extended hinge region in IgA1 which is heavily O-glycosylated compared to IgA2. Both IgA subclasses carry Fc N-glycans at position Asn^{263} and Asn^{459}, in addition IgA2 carries two or three additional glycosylation sites, depending on the allotype [27–29]. In turn, secretory IgA (SIgA) is formed through the translocation of dimeric IgA (dIgA) across mucosal epithelial layers to external secretions, together with the heavily glycosylated secretory component.

Vaccination

The main principle of vaccination, also called immunization, is the induction of a specific antibody mediated immune response that provides protection from infection and disease. The underlying mechanism for acquiring immunity is specific priming of the B-cells by pre-exposure to the antigen or disease-causing pathogen, resulting in the differentiation into specific long-lived plasma or memory B-cells. These specific cells respond quickly upon re-exposure to the priming agent (immunogen), resulting in proliferation and production of high affinity antibodies that specifically target the pathogen. The mechanism of B-cell activation and differentiation is described in more detail earlier in this chapter. Vaccines can be divided into different types, live-attenuated or whole pathogen-preparations, and subunit vaccines. Live-attenuated and whole-pathogen vaccines are basically weakened versions of the pathogen, whereas subunit vaccines consist of inactivated toxins, specific cell-surface proteins and carbohydrates (including bacterial capsular polysaccharides) or conjugate (polysaccharides conjugated to a carrier protein) vaccines [30,31]. Subunit vaccines are usually highly purified components lacking PAMPs, making them weak immunogens, that often require the addition of an adjuvant to initiate an immune reaction that is strong enough to induce long-lasting immunity. Adjuvants improve the recognition of the immunogen by the immune system, eliciting a response resembling a natural innate response. The activation of antigen presenting cells subsequently drive adaptive immunity, resulting in long-lasting immunity. The choice of adjuvant affects the innate response which in turn can direct the adaptive response to the administered immunogen. Commonly used adjuvants include aluminum and oil-in-water emulsions, or combinations of adjuvants that have been specifically designed to drive the wanted response. Thus, it is important to consider the immunogen and the desired immune response when choosing an adjuvant, in order to get a robust response and long-lasting immunity [31,32]. Upon infection the immune response usually results in a polyclonal B-cell activation and expansion, as several different B-cell clones committed to recognizing distinct epitopes (sites of recognition) on the pathogen, resulting in a rapid production of a repertoire of specific antibodies, leading to efficient neutralization and clearance.

2. Glycosylation

Glycosylation is one of the most abundant co-translational and PTM of proteins in general. The two major types of glycosylation, N- and O- linked glycosylation both contribute to important structural, functional and biological properties, including protein solubility, stability, mobility, folding, signal transduction, molecular trafficking and receptor activation. Furthermore, protein glycosylation has also been shown to play a key role in the immune system [33–35]. Given the fact that glycans participate in so many fundamental biological processes it is not surprising that aberrant glycosylation has been associated with numerous congenital, metabolic, neurodegenerative and autoimmune diseases as well as cancer [33,35–42].

Proteins are consistently synthesized as identical copies, determined by the genetic code, through the tightly regulated processes of transcription and translation, followed by precise folding into the correct three-dimensional structure. Glycan assembly is a non-templated process catalyzed through a series of individual reactions. Nevertheless, even though glycan structures are not directly encoded in the DNA, they are determined by the transcription and translation of many hundred glycan related genes encoding an array of glycosidases, glycosyltransferases and various other enzymes and proteins involved in the glycan synthesis processes taking place in the endoplasmic reticulum (ER) and Golgi. The strict donor – acceptor specificity of each glycosyltransferase, meaning that a specific glycosyltransferase can only add one type of sugar residue in a specific linkage, means that the expression of certain glycosyltransferases at any given time indirectly dictates the glycan structures produced [43,44]. As has been shown for IgG, the primary sequence and three-dimensional structure of a protein can sterically hinder the access of the glycosyltransferases resulting in less extended glycoforms [45].

The biochemical process of glycan synthesis

N-linked glycans are exclusively attached to the nitrogen of asparagine residues, specifically within the Asn-X-Ser/Thr motif (where X can be any amino acid except proline) on the surface of the protein. The intricate biochemical process of N-glycan synthesis involves the formation of a lipid-linked oligosaccharide precursor

molecule, the *en bloc* transfer of the carbohydrate to a polypeptide, and the trimming of this precursor molecule to produce the final glycan structure.

The process builds a series of independent reactions, starting with the synthesis of a $Glc_3Man_9GlcNAc_2$ glycan attached to the lipid dolichol (Fig. 2), through a two-step process. First, taking place on the cytosolic side of the ER, is the attachment of the two N-acetylglucosamine (GlcNAc) followed by the first five mannose residues. Secondly, this lipid-liked glycan is translocated to the luminal side of the ER, where the remaining four mannose and three glucose residues are attached. The complete dolichol (lipid)-bound precursor glycan is then transferred to a polypeptide acceptor, a reaction catalyzed by oligosaccharyltransferase (OST). Following transfer to the polypeptide the glycan undergoes extensive, successive, trimming by specific exoglycosidases. Still in the ER lumen, the three glucose residues are hydrolyzed by the enzymes

glucosidase I, removing the first α1-2 linked glucose, and glucosidase II, removing the two inner α1-3 linked glucose residues. The processing continues with the trimming of some or all of the four α1-2 linked mannose residues, hydrolyzed by a series of mannosidases, starting in the ER and continuing through the *cis* Golgi. Some proteins remain in this state, carrying high mannose glycans, and are not processed further. Complex glycan structures continue to be modified as they move through various luminal compartments to the cell surface. These glycan structures are built on a core consisting of three mannose- and two GlcNAc residues. The rebuilding continues in the medial Golgi with the addition of a GlcNAc residue to the 1-3 arm of the core, catalyzed by GlcNAc transferase I, followed by the action of the Golgi mannosidase II catalyzing the removal of two mannosidases on the 1-6 arm of the glycan core. Rebuilding now continues as the protein moves through the medial and *trans* Golgi, with the addition of a GlcNAc residue on the 1-6 arm of the core, catalyzed by GlcNAc transferase II. Further additions of GlcNAc residues, each catalyzed by specific GlcNAc transferases define the branching of the core structure, thus also defining the final glycan structure. The extension of the glycan typically continues with the addition of galactose (often in a β1-4 linkage) to each GlcNAc residues, further extended with sialic acid residues (either in α2-3 or α2-6 linkage), catalyzed by the galactosyltransferase and sialyltransferases respectively [41,46]. Many variations are seen in the terminal end of the N- glycan, however the core can also be differentially modified with the addition of a bisecting GlcNAc to the core mannose residue or by the addition of a core fucose directly linked to the innermost GlcNAc residue. However, the addition of a bisecting GlcNAc hinders the attachment of a core fucose, a property that is being utilized in the pharma industry to design specific antibody glycoforms lacking core fucose. [47–49]. Hybrid glycan structures are produced through alternative processing in the Golgi [50].

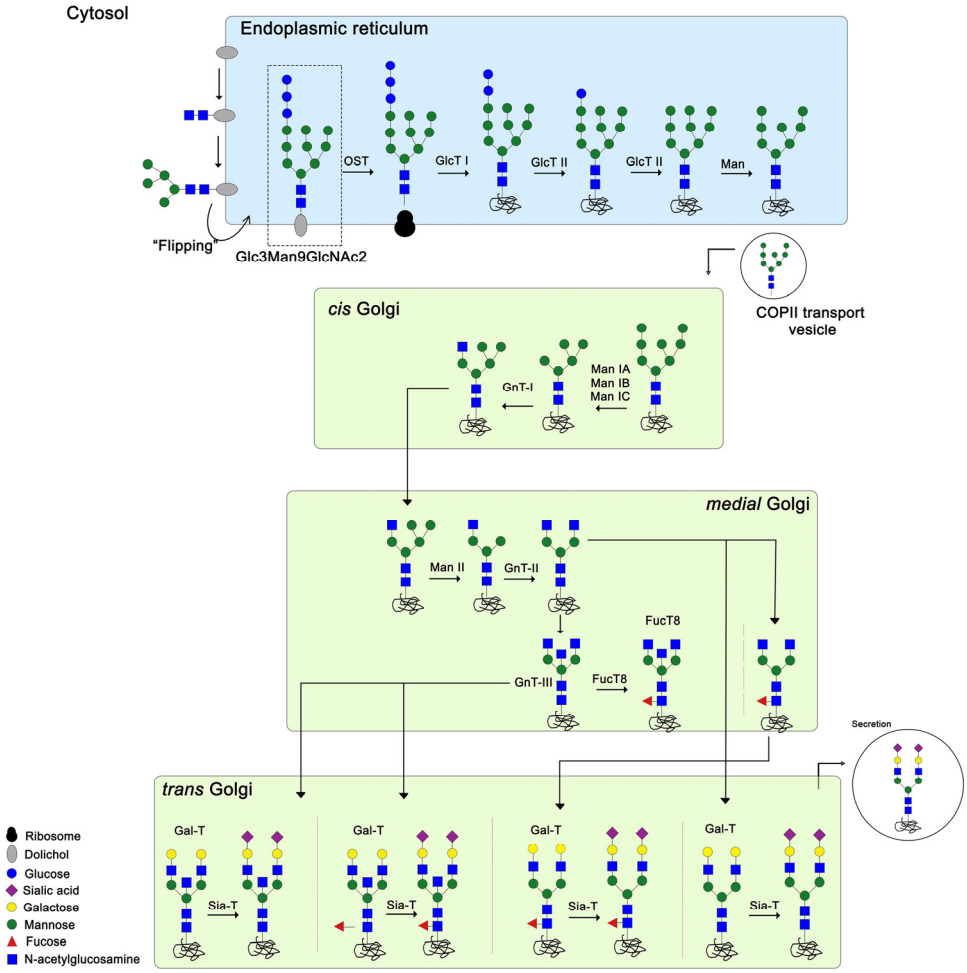

Fig. 2

Schematic figure showing the process of N-glycan synthesis. The N-glycan biosynthesis starts in the endoplasmic reticulum (ER) by the en bloc transfer of a lipid-glycan precursor, $Glc_3Man_9GlcNAc_2$ (comprised of 3 glucose, 9 mannose and 2 N-acetylglucosamine sugar residues) from the dolichol phosphate to Asn, catalyzed by oligosaccharyltransferase (OST). The glucose residues are sequentially removed by two glucosidases (Glc I–II), followed by the removal of an initial Man residue by the ER α-mannosidase (ER α-Man). The glycoprotein is transferred to the the Golgi apparatus for additional trimming by mannosidase I and II (Man I–II) and additional glycan modifications. The glycan is further modified as it moves through the cis- and trans- Golgi, where GlcNAc-transferase I–IV (GnT-I–IV), galactosyltransferases (Gal-T) and sialyltransferase (Sia-T) facilitate further processing generating a wide variety of N-glycoforms. The dynamic biosynthesis of N-glycan is affected by a range of different factors including regulation and gene expression (determining the glycosyltransferase and glycosidase expression levels), the synthesis, transfer and availability of donor nucleotide-activated monosaccharides, the accessibility of the glycoprotein glycosylation sites and epigenetic changes to the glyco-associated genes, pointing out the effect of environmental factors such as age, smoking and diet. Figure modified from Reily, C., Stewart, T.J., Renfrow, M.B. and Novak, J. 2019. Glycosylation in health and disease. Nature Reviews. Neprology 15(6), pp. 346-366.

O-glycosylation is another common protein modification, with an estimated abundance accounting for > 10 % of all glycoproteins. O-glycosylation is initiated by the attachment of the monosaccharide N-acetylgalactosamine (O-GalNAc) or N-acetylglucosamine (O-GlcNAc) to the hydroxyl group of serine or threonine, and possibly tyrosine, residues in the protein, catalyzed by a GalNac (20 homologs) or the GlcNAc transferase (GalNacT and GlcNAcT) respectively. In contrast to N-linked glycosylation, no consensus peptide sequence has been identified for O-linked glycosylation. O-glycans are often bi-antennary structures, generally less branched compared to N-glycans [35,41,51,52]. O-glycosylation has historically been considered a relatively rare PTM, restricted mainly to the modification of mucin or mucin-like proteins. However, more recent glycoproteomic studies has demonstrated that O-glycosylation is abundant, estimating that >80 % of proteins trafficking through the secretory pathway are modified by O-glycans [53,54]. O-glycosylation is a common modification seen on antibodies, in particular on IgA where alterations in O-glycosylation has been associated with IgA nephropathy [55,56]. Recent findings have also indicated the presence of O-glycans in the IgG3 hinge region. Interestingly IgG3 has an elongated hinge region compared to all other IgG subclasses, varying between 27-83 amino acids depending on the IgG3 allotype. This extended hinge confers an increased flexibility between the Fab and Fc regions, as well as a wider and more flexible angle between the two Fab arms which likely results in an increased affinity for divalent binding of certain antigens [57]. The functional role of IgG3 hinge O-glycosylation has not been established, however it has been suggested to support the extended hinge conformation and thus contribute to the flexibility and orientation of the Fab fragments. It has also been suggested to protect against proteolytic degradation, as the Ig-hinge region is a target of many bacterial or endogenous proteases, and the extended IgG3 hinge would presumably make it more susceptible to proteolytic degradation. Finally it could be speculated that deviations in the O-glycosylation of IgG3 may have pathological effects, as has been described for IgA nephropathy [57]. Furthermore, recent glycosylation analysis of therapeutic Fc-fusion proteins produced in mammalian cell expression systems demonstrated that many of these proteins were modified by O-glycosylation. This unexpected observation could very well have implications in regards of efficacy and safety [58]. The implications of glycosylation in regards to the safety and efficacy of biologics will be discussed further in chapter 4.

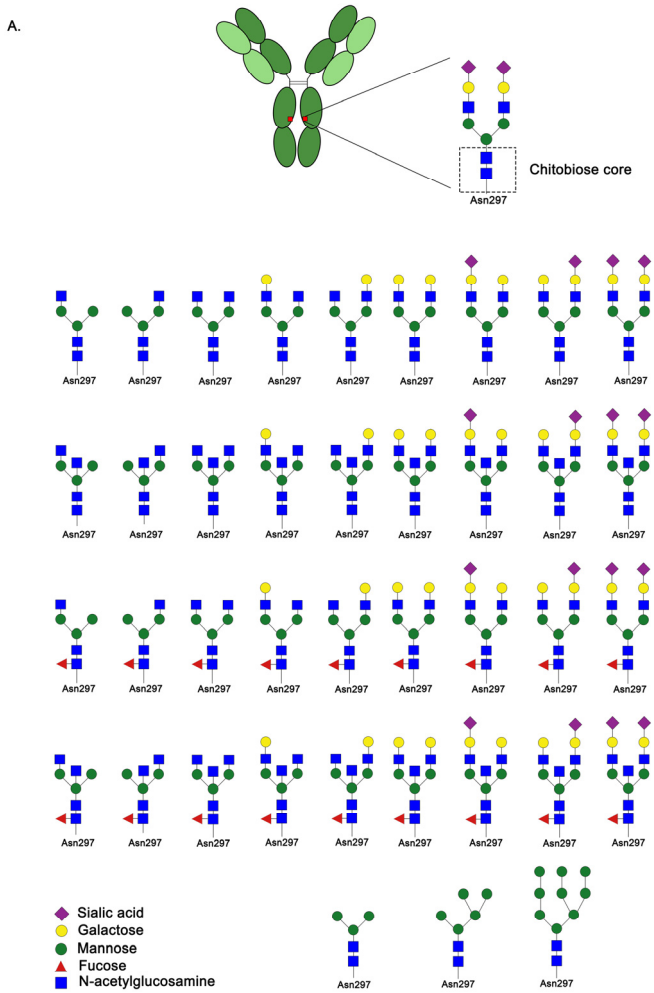
A.
Chitobiose core
Asn297
Sialic acid
Galactose
Mannose
Fucose
N-acetylglucosamine
B.

Fig.3.
Figure showing the theoretical IgG N-glycoforms attached to Asn297 in the CH2 domain, and the distribution of IgG glycoforms in normal serum. A) A schematic representation of the theoretical IgG Asn297 N-glycoforms. The common N-glycan chitobiose core (Man3GlcNAc2) is denoted with the lined frame. The figure demonstrates the theoretical N-glycan structures, albeit the theoretical number of IgG variants increases to 144 when considering that there are four IgG subclasses, and the IgG variants exceed 400 when considering that the two CH2 domains can be differently glycosylated. Removal of core fucose drastically increases IgG affinity for FcγIIIa leading to enhanced antibody dependent cell-mediated cytotoxicity (ADCC), increased sialylation of the conserved Fc glycan has been associated to increased anti-inflammatory properties of IgG and terminal galactose has in turn been associated and enhanced C1q binding and anti-inflammatory properties when present in immune complexes. B) HPLC of IgG N-glycans, released with PNGaseF followed by 2-AB labeling. The chromatogram displays the distribution of IgG glycoforms in the sample from a healthy donor. Peak height represents relative abundance of the respective glycoform. Glycan structures annotation; blue square: N-Acetylglucosamine, green circle: mannose, yellow circle: galactose, purple diamond: sialic acid, red triangle: fucose.

The process of glycan biosynthesis is best described as dynamic, involving hundreds of enzymes and other proteins. This means that the final outcome of a glycosyl reaction is affected by a range of different factors including genetic mutations and polymorphisms; regulation of gene expression; the synthesis, transfer and availability of donor nucleotide-activated monosaccharides; the efficiency of glycosyltransferases or glycosidases; the accessibility of the glycosylation site; and the competition between different glycosyltransferases. Furthermore, mutations in the gene encoding the glycoprotein (eliminating or generating additional glycosylation sites or altering protein conformation), as well as the local environment and signaling during B-cell activation can also influence the final glycoprotein product [43,59–62]. Adding to the complexity are the potential epigenetic changes to the glyco-associated genes which indirectly shows the importance of environmental factors including physical, nutritional and behavioral factors such as age, smoking, diet, weight, infection and hormonal changes [40,59,63–67]. All these factors affect the final glycan structure, which ultimately affects the properties of the glycoprotein. Only for IgG there are 36 theoretical glycovariants, out of which 30 have been identified by mass spectrometry, all with different effects on complement activation, receptor affinity, and mediating distinct downstream effector functions, indicating the impact and effect of glycosylation [68]. Theoretical N-glycan structures and the distribution of the different IgG glycoforms in human blood, are shown in in Fig. 3. This is discussed in more detail further on in this chapter.

In addition to protein glycosylation along the secretory pathway, the presence of extracellular glycosyltransferases and the notion of extrinsic glycosylation has been recognized for decades, however the significance and implications of this process has long remained unknown. Recent research has now clearly established that extrinsic posttranslational remodeling of glycoproteins is an important physiological process that can remodel both cell surface and secreted glycans *in vivo*. Platelets have been identified as a main reservoir for both glycosyltransferases and substantial levels of activated–sugar substrates, which are released upon platelet activation [69–72]. This alternative glycan remodeling process has also been shown

to remodel antibodies in circulation, for example resulting in B-cell independent sialylation of the Fc glycan on IgG, which is thought to contribute to immunosuppressive activity [69,70,73,74].

Antibody glycosylation in immunity

The first detailed investigations looking into antibody glycosylation dates back to the 1980's, a decade of pioneering research during which the first crystal structure and refined model of the IgG Fc-fragment was also elucidated [75]. In the following decades accumulating structural, biochemical and biological evidence established the importance of antibody glycosylation for antibody structure and function. This accumulated knowledge has been successfully applied to develop advanced methods of antibody engineering, allowing specific tailoring and fine-tuning of antibody functionality, which in turn is used for the development of sophisticated antibody-based therapies. The development of therapeutic antibodies and methods of glycoengineering is addressed in more detail in chapter 4.

There is considerable diversity in the location and number of N- and O- linked glycosylation between the different antibody isotypes. Heavy chain glycosylation accounts for 2-3% of the IgG molecular weight, compared to 12-14% of IgM, IgD and IgE [19]. Importantly glycosylation is essential for antibody functionality, and modifications in the glycan structure can direct and fine-tune the induced immune response [76]. However, although the importance of IgG Fc glycosylation is well understood, the antibody repertoire includes additional isotypes and subclasses, and much is still unknown about the functional role and the importance of antibody glycosylation.

IgG glycosylation

IgG is by far the best studied antibody isotype and the conserved N-glycan attached to Asn-297 in the CH2 domain of the antibody has been the focus of extensive studies for decades. In healthy individuals IgG is predominantly represented by core fucosylated (96 %), bi-antennary complex type structures. The glycan is composed of a heptasaccharide core constructed of four GlcNAc and three mannose residues ($GlcNAc_2Man_3GlcNAc_2$), which is further extended with one (predominantly in the α1-6 arm) or two galactose residues (40%). A minor proportion of the glycans are further modified by the addition of one or two sialic acid residues (4 %), and bisecting GlcNAc (8 %) [12,19,68,77,78]. The theoretical number of possible IgG variants increases to 144 when taking into account that there are four IgG subclasses, and when considering that the two CH2 domains can be decorated with different N-

glycans the number of potential IgG glycovariants exceeds 400 [68,79]. This conserved N-glycan contributes to the stability and function of the IgG Fc and is required for antibody – receptor interactions, and modification of the glycan structure results in an altered binding [19,76,80–82]. The removal of the glycan by glycosidases, or through mutation of the glycosylation sites on the protein drastically reduces binding [76,81].

The IgG Fc N-glycan composition not only contributes, but can also define the antibody mediated immune activation, giving the antibody pro- or anti-inflammatory properties. For example the removal of the core fucose drastically increases the affinity (up to a 100 fold) for the low affinity receptor FcγRIIIa, resulting in enhanced ADCC [83–86]. Moreover, increased sialylation of the conserved Fc glycan has been associated to increased anti-inflammatory properties of IgG, however the findings are controversial and the mechanisms underlying this activity remain elusive (Fig. 4). The C-type lectin DC-SIGN has been identified as the receptor involved in mediating the anti-inflammatory properties of hypersialylated IgG, believed to suppress inflammation through induction of the inhibitory IgG receptor FcγRIIb. However, several research groups are presenting considerable amounts of contradicting data, disputing both the anti-inflammatory properties of sialylated IgG and the role of DC-SIGN [87–91]. Furthermore, hypersialylation of this N- linked glycan has also been suggested to prolong antibody serum half-life. The underlying mechanism for this prolongation of IgG serum half-life is still not clear, however it is thought to be FcγRn and FcγR independent and rather mediated through the masking of the galactose residues by sialic acid, hindering antibody binding to the asialoglycoprotein receptor in the liver, salvaging it from degradation [88]. The exact role for IgG galactosylation and its influence on IgG activity is not as clear. However, recent reports have indicated an association between highly galactosylated IgG and enhanced anti-inflammatory properties when present in immune complexes [78,92]. Aberrant IgG glycosylation, often referring to increased amounts of agalactosylated IgG – resulting in a shift towards a highly inflammatory glycosylation profile, is closely associated with certain diseases including rheumatoid arthritis (RA), Crohn's disease, SLE and certain lymphomas [19,93–96]. As of yet, bisecting GlcNAc has not been recognized to directly influence the effector functions of IgG, however as it hinders the biosynthetic process of core fucosylation it indirectly enhances ADCC activity [47,49,97,98]. Interestingly, this knowledge has resulted in advanced methods of glycoengineering that is now being applied for the design of novel therapeutic monoclonal antibodies (mAbs) and vaccine design.

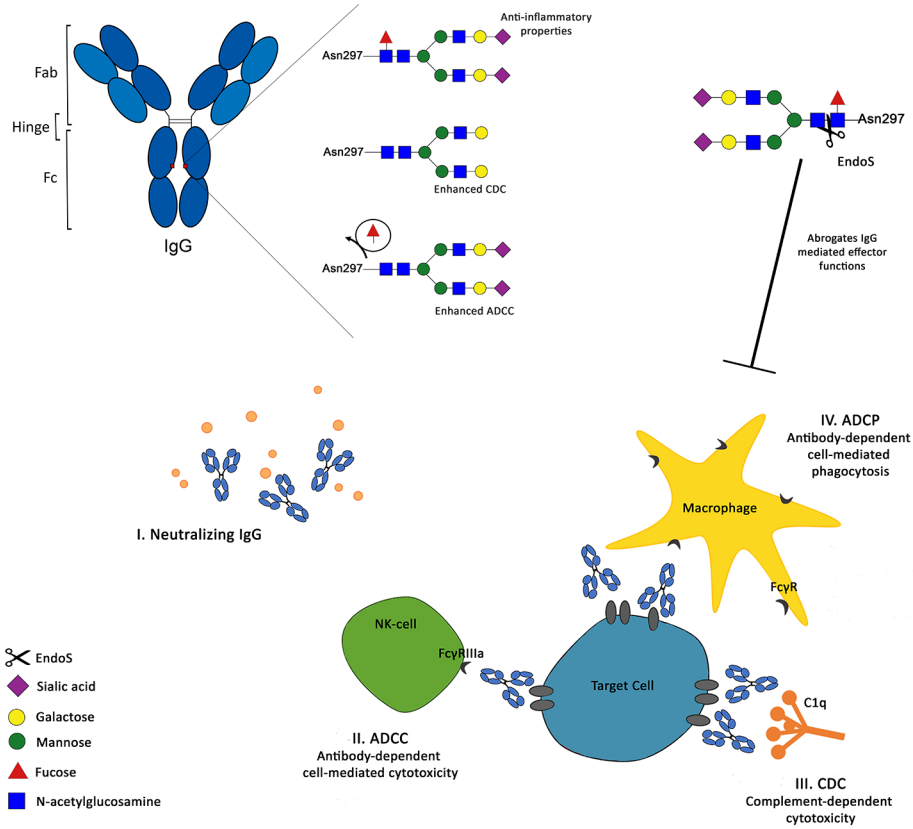

Fig. 4
A Simplified schematic overview of antibody mediated defense mechanisms including the effects of IgG Fc N-glycan modifications on the induced effector functions. Beyond their role in neutralization and clearance, mediated through Fab binding of antigens (I), antibodies also mediate effector functions through their Fc-region. Bridging of immune effector cells, including NK-cells, with target cell induces antibody-dependent cell-mediated cytotoxicity (ADCC) resulting in direct killing of the target cell (II). Removal of the core fucose on the IgG Fc N-glycan greatly enhances the affinity for the activating FcγRIII on NK-cells resulting in enhanced killing of the target cell. Binding of C1q to opsonized target cells induces complement-dependent phagocytosis (CDC) (III). Terminal galactosylation of the IgG Fc N-glycans is associated with enhanced CDC. Target cell opsonization with IgG also mediates antibody-dependent mediated phagocytosis (ADCP) or ADCC by macrophages. Hydrolysis of the conserved IgG Fc N-glycan by the streptococcal enzyme EndoS (IV) results in abrogation of antibody mediated effector functions, however the capacity to neutralize and clear infectious agents/ antigens through Fab-binding is retained.

In addition, 15-25 % of serum IgG are estimated to carry N-glycans in the variable, antigen binding domains. These glycans are in general represented by bi-antennary, complex type glycans with increased levels of galactosylated, sialylated and bisecting glycan structures, with reduced fucosylation, as compared with Fc N-glycans [93,99]. The importance and function of Fab glycosylation has long been overlooked and regarded as less important. However recent reports point to a functional role of Fab glycosylation, showing associations between enhanced Fab glycosylation and several autoimmune diseases and certain forms of B-cell

lymphoma. These associations indicate that general shifts in IgG Fab glycosylation patterns might be linked with disease, however any precise associations or glycosylation patterns still remain to be elucidated. Functionally, changes in IgG Fab-glycosylation has been shown to differentially affect antigen binding affinities, antibody serum half-life, antibody aggregation and complex formation. Recent reports have also suggested that IgG Fab sialylation contributes to the anti-inflammatory properties of IVIg (intravenous immunoglobulin). However these findings have been contradicted in other studies, and further research is needed to clarify the role of Fab sialylation in antibody mediated immunity [93,100,101].

IgA glycosylation

Structurally IgA glycosylation has been well characterized, demonstrating numerous potential and occupied N- and O-linked glycosylation sites, the exact number and structure, depending on the IgA subclass and allotype. However, the functional importance of IgA glycosylation in immunity is not as clear. The statements are restricted to serum IgA, which differs significantly from secretory IgA. Structural differences between the IgA subclasses include an extended hinge region in IgA1, compared to IgA2, which is heavily O-glycosylated. Both IgA subclasses consistently carry two N-glycans located in the CH2 domain and the IgA Fc-tail. IgA2 carries an additional two or three glycosylation sites, depending on the allotype, located in the CH1 and CH2 domains [27–29]. IgA glycosylation is described to be predominantly represented by digalactosylated biantennary complex type glycans, with a high amount of terminally sialylated glycoforms (>90 %), in contrast to IgG where only a minor fraction (5-10 % mono-, and 1% disialylated) of the antibodies are sialylated [28,29,102]. The glycosylation in the CH2 domain of IgA, corresponding to the conserved IgG Fc N-glycan, has been shown not to be critical for interactions between IgA and FcαRI [27,28]. However, IgA glycosylation contributes to the immune defenses by other means, for example by interfering with, and abrogating, cell surface attachment of influenza and other enveloped viruses [103].

Antibody mediated pathologies

Under normal circumstances antibody mediated immunity is essential for a well-functioning immune system, protecting us from invading microorganisms and other foreign antigens. However, there are instances where the antibodies react with self-molecules, becoming autoantibodies, resulting in the development of autoimmune diseases such as RA, SLE, thrombocytopenic purpura, autoimmune hemolytic anemia or Graves' disease [104]. The global prevalence of autoimmune diseases has

increased during the past decades, representing a major disease burden worldwide. Since the first observations during the 1970s there has been an accumulation of evidence showing an association between aberrant IgG glycosylation and a range of different inflammatory, autoimmune diseases. The best described association between disease and abnormal glycosylation, is the skewing towards agalactosylated (IgG-G0) glycan structures showing a significant correlation between increased agalactosylated IgG and RA disease severity [94,104,105]. The underlying mechanisms driving this shift in glycosylation and whether or not these findings can be used as biomarkers for certain autoimmune conditions remains to be determined.

Fc receptors

It is becoming evident that the importance of glycosylation for antibody mediated immunity extends beyond the importance of the conserved N-linked glycan on IgG. Recent discoveries indicating functional roles of both Fab- and Fc receptors (FcRs) glycosylation in immunity, are revealing a much more complex and dynamic role of glycosylation in modulating the immune response.

Together with the immunoglobulins the FcRs are critical components of the immune system, linking the adaptive antibody response and the innate effector functions. These complex glycoproteins comprise an array of receptor types, displaying high variability in their distribution on different immune cells including monocytes, neutrophils, B-cells, macrophages, natural killer (NK) cells and platelets (table 1) [106]. The classical Fc receptors usually referring to FcγR, FcαR, FcεR, FcδR and FcμR display a restricted antibody isotype specificity and mediate distinct immune responses following activation, including effector functions such as phagocytosis, activation of the classical complement pathway and ADCC. The neonatal Fc receptor (FcRn) is involved in placental transport of IgG from mother to fetus and in IgG recycling, prolonging serum half-life and maintaining high IgG concentrations in circulation. Additional FcRs include the pIgR (polymeric immunoglobulin receptor), TRIM21 (Fc Receptor tripartite motif containing-21), Fc receptor like proteins, a number of siglec receptors, and DC-SIGN. The pIgR mediates transcytosis of polymeric IgA and IgM from the tissue to the mucosal layers in the luminal space, contributing to mucosal immunity. TRIM21 is a cytosolic FcR expressed by all cells, which upon engagement mediates antibody dependent cellular neutralization of intracellular pathogens (ADIN) [107–110].

Table 1. Human FcγRs

Representation of human classical and non-classical IgG receptors. The classical human FcRs consist of FcγRI, FcγRIIa/b/c and FcγRIIIa/b, whilst the non-classical receptors include FcγRn, TRIM-21 and DC-SIGN. The table describes the receptor activity (activating/ inhibiting), distribution of the receptors on different cell types, variations in IgG subclass binding (ordered according to decreasing affinity).

	Receptor	Function	Affinity	IgG subclass	Expression
Classical FcRs	FcγRI	Activation	High affinity -Binds monomeric IgG	IgG1 IgG3 IgG4	Monocytes Macrophages Dendritic cells <u>Inducible</u> Neutrophils Mast cells
	FcγRIIa	Activation	Low affinity -Only binds immune complexes	IgG1 IgG3 IgG2 IgG4	Monocytes Macrophages Neutrophils Mast cells Basophils Eosinophils Platelets
	FcγRIIb	Inhibition	Low affinity -Only binds immune complexes	IgG1 IgG3 IgG4 IgG2	Circulating B-cells Basophils Monocytes (20%) Neutrophils (4%) Macrophages Dendritic cells
	FcγRIIc	Activation	Low affinity -Only binds immune complexes	IgG1 IgG3 IgG4 IgG2	NK-cells Monocytes Neutrophils
	FcγRIIIa	Activation	Low affinity -Only binds immune complexes	IgG3 IgG1 IgG4 IgG2	NK-cells Monocytes Macrophages Neutrophils
	FcγRIIIb	Activation	Low affinity -Only binds immune complexes	IgG3 IgG1	Neutrophils (selectively by 20-30% of humans) Basophil (subset)
Non-classical FcRs	FcRn	-Recycling -Placental transport	High affinity <pH 6.5	IgG1 IgG2 IgG3 IgG4	Epithelial cells Endothelial cells Macrophages/monocytes Dendritic cells Neutrophils(intracellular)
	TRIM 21 (intracellullar)	Activation	High affinity (dimeric form)	IgG1-4 IgM IgA	Ubiquitous in most tissue cells
	DC-SIGN	Activation	C-type lectin	Glycoproteins -fucose -mannose	Dendritic cells (subsets) Inflammatory macrophages

FcγRs, belong to the immunoglobulin-like superfamily, are typically single pass transmembrane glycoproteins, expressed on various immune cells, generally acting through the immunoreceptor tyrosine-based activation (ITAM) or inhibition (ITIM) motifs. There are five classical activating IgG receptors FcγRI, FcγRIIa, FcγRIIc,

FcγRIIIa and FcγRIIIb, and one inhibitory receptor FcγRIIb all displaying unique binding profiles to each of the different IgG subclasses. The FcγRI is characterized by its high affinity for IgG1 and IgG3, displaying reduced binding of IgG4 and a complete absence of IgG2 binding. The high FcγRI affinity (10^8-10^9 M^{-1}) enables binding of monomeric IgG, in contrast to the low affinity receptors FcγRIIa/ IIc and FcγRIII which require interactions with immune complexes for activation [68,107,111]. Importantly the high and low affinity, activating and inhibitory receptors, are not only distributed differently on different cells but they are also co-expressed to varying extents. The co-expression of activating and inhibitory receptors creates a threshold, regulating and balancing the activation and extent of the immune response. Imbalanced immune responses, caused by dysregulation of pro-inflammatory signaling and /or the loss of inhibitory signaling is associated with autoimmune diseases such as arthritis, multiple sclerosis (MS) and SLE [111,112]. Studies have shown that a deletion of the inhibitory Fc receptor in mice, or downregulation in FcγRIIb cell surface expression on activated B-cells in mice and humans, results in loss of tolerance and is strongly associated with the development of autoimmune diseases [113–115]. This stresses the importance of a tight and accurate regulation of the activating signaling pathways that drive potent and potentially harmful pro-inflammatory responses.

Fc-receptor glycosylation in immunity

Fc-receptor glycosylation has proven to be far more intricate to study compared to that of antibodies, and the available information is still rather limited. What has been established is that all the FcRs have multiple potential glycosylation sites, with varying numbers and locations depending on the receptor type. Receptor glycosylation has also been shown to be cell type specific, adding an additional factor contributing to the complexity of FcR glycosylation. It has been suggested that a rapid upregulation of FcRs upon cell activation would result in the alteration of the glycosylation profile, possibly promoting antibody binding, as compared to a resting cell where the glycosylation would promote dissociation. Thus, any detailed information to the exact occupancy and constitution of these glycan structures, as well as their role in FcR biology, is still largely unknown [19,107]. As with general protein glycosylation, the glycosylation of FcRs also contributes to folding, stability and protection from proteolytic degradation, thus not all glycans must be involved in antibody interactions. The functional importance of FcR glycosylation in regards to antibody interaction is perhaps best exemplified by the finding that the removal of the N-linked glycans at Asn162 in the low affinity FcγRIIIa results in a significant reduction in IgG_1 binding, showing that these glycans directly regulate binding of IgG. The FcγRIIIa N-glycan at Asn45 also showed to have an inhibitory effect on

IgG binding [86,116]. It should be noted that, due to the difficulties in obtaining sufficient amounts of material for analysis, most of what is known for FcγRs is based on research with recombinantly expressed proteins and very little is known about the glycosylation of Fc-receptors in their natural states, bound to cell surface membranes of various immune cells. FcαRI, the specific receptor for IgA1 and IgA2, is believed to be heavily glycosylated, with six potential N-linked glycosylation sites and seven potential O-linked sites. As with the other FcRs there is little information about the exact composition and biological function of the majority of these glycans, however mutagenesis studies, specifically altering FcαR glycosylation demonstrated changes in IgA affinity, indicating that the different glycans play a role in the IgA interaction.

Antibodies and mouse models

The usefulness of animal models is a constant topic of debate questioning the translatability of the results obtained in for example a mouse to humans. However, it cannot be ignored that the use of animal models has contributed greatly, enabling major advances within both basic science and medical research. One of the best established and most widely used model animals in scientific research is the mouse, thus this discussion will be limited to mouse models specifically focusing on antibodies and FcRs. Importantly, mouse models are commonly used in research to address a variety of scientific questions and it is of utter importance to understand the discrepancies between human and murine Ig's and FcRs as their expression patterns and antibody binding abilities are quite distinct (table 1, 2) [112,117,118]. For example, in regards to expression of FcγRII, mice only express the inhibitory FcγRIIb. Thus, despite the comparable expression patterns of FcγRIIb on various cell types in mice and humans the lack of the activating FcγRII receptor may result in differences in the balance between activating and inhibitory signalling. Although the lack of the activation FcγRII might be compensated by another activating receptor, this difference could have implications that might need to be considered when deciding on the experimental setup, for the interpretation of the results, and for the translation to human physiology [118,119].

The detailed information, and availability of the complete human and mouse genome has shown a remarkable genetic homology between the two species. The similarities in biochemical pathways and physiological functions, as well as the opportunities for gene manipulation, has further prompted the use of the mouse as an experimental system. Methods of genetic manipulation, including the creation of transgenic-, knockout- and knockin mice has provided a powerful tool to investigate a wide range of scientific questions ranging from basic science, elucidating underlying genetic and biological mechanism related to disease, to the development

and evaluation of novel therapies and vaccines. However, there are important genetic differences between the two species, which except for the obvious differences in size, life span, microbiome and metabolism, include genetic redundancies and regulation of gene-expression levels, which translate into physiological differences. Laboratory mice used for research have been developed as inbred strains with highly homogenous genetic compositions, which eliminates an unpredictable variable and increases the reproducibility of the results. However, some genetic and physiological variations within each species and between different mouse strains need to be considered when choosing the most appropriate mouse strain for the particular experimental setup, as this can affect the outcome of the results [120–124]. Although the results acquired in an animal model are not always directly translatable to humans, the studies on mice has contributed immensely to the understanding of human biology.

Table 2. Mouse FcRs

Mouse IgG receptors. The mouse FcγR consist of four members distinguished by their activity, distribution on different cell types, ability to bind monomeric IgG vs. immune complexes and affinity for the different IgG subclasses (ordered according to decreasing affinity).

	Receptor	Function	Affinity	IgG subclass	Expressed on
Classical FcRs	FcγRI	Activation	-High affinity -Binds monomeric IgG2a	IgG2a	Monocytes Macrophages
	FcγRIIb	Inhibition	-Low affinity -Only binds immune complexes	IgG1 IgG2a IgG2b	B-cells Monocytes Macrophages Dendritic cells Basophils Eosinophils Mast cells
	FcγRIII	Activation	-Low affinity -Only binds immune complexes	IgG1 IgG2a IgG2b	NK-cells Monocytes Macrophages Dendritic cells Basophils Eosinophils Mast cells
	FcγRIV	Activation	-Intermediate -Only binds immune complexes	IgG2a IgG2b (IgE)	Monocytes Macrophages
Non-classical FcRs	FcRn	-Recycling -Placental transport	High affinity <pH 6.5	IgG1 IgG2a IgG2b IgG3	Placental tissue Epithelial cells Endothelial cells Macrophages
	DC-SIGN (8 homologs)	Activation	C-type lectin	Glycoproteins	Dendritic cells Monocytes/macrophages

3. Antibody modulation and bacterial virulence

Bacteria have through co-evolution with the human host developed numerous strategies of immune evasion, including several mechanisms to neutralize antibody mediated immunity. Such mechanisms include the use of decoy antigens, rapid mutation of highly immunogenic epitopes e.g. the O-antigen on lipid A of LPS, antibody binding by the Fc-region, proteolytic cleavage of the protein into smaller fragments, or through modulation of the antibody glycosylation. *Streptococcus pyogenes* is well adapted to the human host and has developed numerous strategies of immune evasion. Likewise, *Bdellovibrio bacteriovorus,* a predatory bacterium, non-invasive to eukaryotic cells, has adapted to its environment and produces a plethora of proteolytic proteins to survive and thrive. This chapter introduces these bacteria, focusing on their means of modifying or degrading antibodies.

Streptococcus pyogenes

Streptococcus pyogenes, or Group A Streptococcus (GAS), is a Gram-positive, strictly human pathogen responsible for causing a variety of diseases ranging from mild tonsillitis and impetigo to life-threatening invasive infections such as necrotizing fasciitis, bacteremic pneumonia, sepsis and streptococcal toxic shock syndrome. GAS infections can also lead to serious antibody mediated post-streptococcal sequelae including acute rheumatic fever (ARF) and post-streptococcal glomerulonephritis, that in turn can lead to rheumatic heart disease and chronic kidney disease. With a global prevalence of severe GAS infections exceeding 18 million cases, with approximately 1.8 million new cases/ year and accounting for 500 000 deaths annually, GAS remains a major cause of morbidity and mortality worldwide [125–128].

With no other natural host, this streptococcus has adapted to transmission, within human populations, benefitted from crowding and limited access to hygiene primarily making it a disease associated with poverty. Luckily GAS retains a susceptibility to penicillin and other antibiotics, and the disease epidemiology and prevention strategies are well understood [125,126,129]. GAS is primarily localized

on epithelial surfaces of the oral-nasal mucosa and skin, and has been shown to colonize large parts of the population. Several epidemiological studies have shown that asymptomatic carriage is a common phenomenon, with numbers ranging between 5-20% in schoolchildren [130–132]. The underlying molecular mechanisms enabling the bacteria to peacefully coexist for prolonged periods, rather than causing disease, are still poorly understood. Streptococcal disease commonly arises from an initial colonizing state, thus it must be considered that this shift from coexistence to pathogenesis is caused by a disturbance in the balance between the microorganism and host. Such disturbances can often be attributed to factors including preceding or concurrent virus infections and other changes in the local environment [133].

GAS strains are defined by the group A carbohydrate on their surface and further classified based on differences in the *emm* gene encoding the dominant surface antigen and important virulence determinant, the M protein [129,134]. The M-protein serves many functions, contributing to GAS virulence, including resistance to phagocytosis and antibacterial activity of histones, contributing to adherence and intracellular invasion [135–137]. There are > 150 GAS strains, based on serological M-types (> 100 according to the Center for Disease Control Prevention [138]), and > 250 *emm* types, defined by sequencing of the hypervariable region at the 5' end of the M-protein gene *(emm)*, displaying large geographic variations in the distribution. Interestingly, certain *emm* types are more predominant and have been distinctly associated with certain infections, e.g. M1 and M3 are viewed as hypervirulent and are strongly associated with severe invasive infections [139–144]. A direct correlation between the presence of type-specific anti- M-protein antibodies and a resistance to GAS infections was shown as early as in the 1960s [145]. Co-evolution with the human host has rendered GAS well adapted, with a large array of virulence factors involved in host-pathogen interactions facilitating colonization and long-term survival, infection and immune evasion. These virulence factors include several different adhesins, in addition to the M-protein, enabling the attachment to host tissues, factors for evasion of phagocytosis, and factors facilitating invasion or movement through tissue.

This array of virulence factors is regulated through several stand-alone multigene regulons that respond to changes in host environments through individual transcriptional regulator proteins. Three of these regulons, Mga, RofA-like protein (RALP) and RopB, have been extensively characterized in regards to their role in GAS pathogenesis, however much is still unknown about how these regulons sense environmental changes. GAS also utilizes several two-component transduction regulatory systems to sense and respond to specific environmental changes, and one of the best described transcriptional regulator in GAS is the two-component CovRS system (control of virulence). This signal transduction system directly or indirectly regulates 15% of the GAS genome [146]. CovR acts primarily as a repressor of

transcription, directly regulating several important virulence genes including *ska* (streptokinase), *sagA* (streptolysin S), *speB* (cysteine protease), and those of the *has* operon coding for genes essential for synthesis of the hyaluronic acid capsule. Bacteria respond to stress by altering their gene expression to adjust for growth in the new conditions, e.g. increased temperature resulting from fever during an infection, and CovRS has been deemed essential for GAS to survive during stress. CovS is believed to act as a bifunctional sensor (acting both as a kinase and a phosphatase) acting as a phosphatase in response to stress, relieving CovR-P mediated repression, resulting in the expression of CovR repressed genes [147–150]. Additionally, CovR has been shown to regulate genes involved in basic metabolic processes including carbohydrate catabolism and nitrogen utilization, displaying a close link to the catabolite control protein A (CcpA). CcpA is a stand-alone regulatory protein which globally regulates transcription in response to carbohydrate availability, but has recently also been shown to contribute to GAS virulence. The comparison between GAS CovR and CcpA transcriptomes displayed significant overlaps, including both *speB* and *ndoS,* indicating that they influence many of the same genes and appear to cooperate during GAS infection (Fig. 5) [150–152]. The deletion of CovR, CovS and CcpA can result in significant change in GAS virulence, and it has become evident that CovR/S as well as CcpA function is an important regulator during GAS infection. This has been extensively investigated using mouse models of invasive disease, and different ΔCovR (CovR deletion) GAS strains. The results demonstrate enhanced virulence and strain specific differences, thus in some strains CovR deletion increased lethality, while in others the CovR deletion reduced lethality but resulted in increased soft tissue damage. Additionally, GAS retrieved from mice with severe invasive infection displayed mutations in CovRS suggesting that these mutants are selected for and that CovRS regulation is important for the transition from local soft-tissue to systemic dissemination during invasive GAS infection. Interestingly, CcpA has been shown to strongly contribute to the generation of spontaneous *covRS* mutations during infection, further emphasizing the interplay between these systems and their contribution to GAS pathogenesis [148,150,153,154].

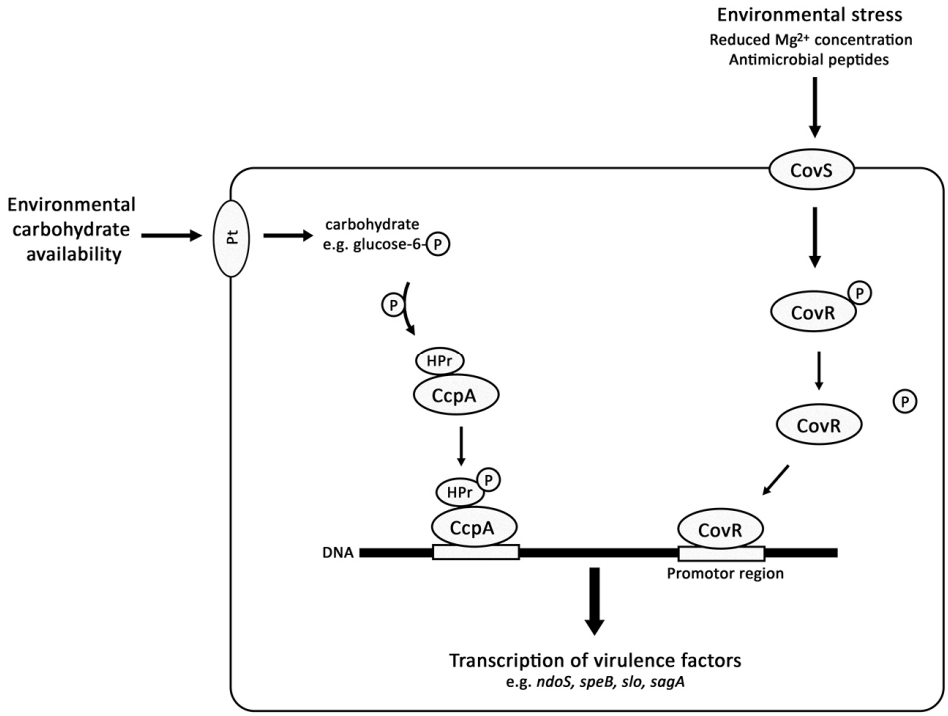

Fig 5
Model showing CcpA and CovR/S gene regulation and contribution to Streptococcus pyogenes virulence. The two-component gene regulatory system CovRS (Control of virulence) and the stand-alone global regulatory protein CcpA (catabolite control protein A) regulate transcription of S. pyogenes virulence genes in response to environmental stressors and carbohydrate availability, respectively. CovR/S and CcpA display transcriptional overlaps, regulating the expression of virulence genes such as ndoS, speB, slo and sagA, suggesting potential cooperation and redundancy during S. pyogenes infection. Abbreviations in the figure: phosphotransferase (Pt), phosphocarrier protein (HPr). HPr is a component in the the sugar transport and phosphorylation system – phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) – used by numerous Gram-positive and Gram- negative bacteria.It has also been clearly demonstrated that the transcription levels of CcpA and CovRS influenced genes are affected by environmental factors, differing greatly between planktonic bacterial growth in standard Todd-Hewitt (THY) medium compared to the transcription during infection. The transcription of certain CcpA and CovRS regulated genes, encoding well known GAS virulence factors, are either repressed during growth in THY or activated during infection, showing that CcpA and CovRS gene expression differs significantly depending on the surrounding environment [150].

Streptococcus pyogenes antibody modulating enzymes

As previously described antibody mediated immunity plays an important role in the defense against invading microorganisms, thus it is not surprising that GAS virulence factors also include specific antibody degrading or modulating enzymes. The large number of GAS serotypes, entailing a large surface antigen variability, as well as strategies to counteract host adaptive immunity often renders the protective immunity against GAS somewhat insufficient [145].

IdeS (immunoglobulin G degrading enzyme of *Streptococcus pyogenes*) and SpeB* (streptococcal cysteine protease exotoxin B) are secreted cysteine proteases, both regarded as streptococcal virulence factors. IdeS specifically cleaves the IgG HC just below the hinge region, resulting in an intact F(ab)2 fragment and two Fc-fragments [155,156]. IdeS-mediated hydrolysis of IgG eliminates antibody mediated effector functions, but does not necessarily abrogate all antibody functions. Studies have shown that $F(ab)^2$ and Fab fragments, without the Fc, can retain their capability to neutralize viruses and bacteria by hindering attachment and invasion of host cells, and through the neutralization or inhibition of microbial toxins. However, so far many of these studies showing positive results *in vitro* have not conferred protection in animal models of infection [157–160]. SpeB is a more promiscuous, multifunctional protease, acting on a number of substrates including IgG, well-known to contribute to *S. pyogenes* pathogenicity. However it has recently been argued that SpeB does not naturally hydrolyze IgG *in vivo* under physiological conditions [154,161–163]. EndoS (Endoglycosidase S) and EndoS2 are two streptococcal endoglycosidases, belonging to the glycoside hydrolase 18 (GH18) family, that specifically cleave the conserved Fc N-glycan at position Asn297 on native IgG. More specifically these enzymes cleave the glycan between the two GlcNAc residues in the chitobiose core, leaving IgG with only the innermost GlcNAc or a GlcNAc with an attached fucose residue (Fig.1), displaying slight discrepancies in their substrate preferences. Compared to EndoS, EndoS2 has a somewhat broader substrate specificity also hydrolyzing high-mannose and hybrid-type glycoforms as well as an additional substrate, native AGP (α_1-acid glycoprotein) [164–166]. It is well known that hydrolysis of the conserved N-linked glycan on IgG results in the impairment of IgG mediated effector functions and we recently showed, for the first time, that EndoS is active and specifically hydrolyzes the Asn297 N-linked glycan on IgG *in vivo* during natural *S. pyogenes* infection in human patients. Using a mouse model of invasive *S. pyogenes* infection we could show that EndoS contributes to pathogenesis in the presence of adaptive immunity [167]. This confirms that bacterial antibody modulation occurs during ongoing *S. pyogenes* infection, and these bacterial strategies of immune evasion should be taken into consideration as they potentially could have implications for the treatment of severe *S. pyogenes* infections, and for the efforts at vaccine development [168].

Other EndoS-like enzymes including EndoSD from *Streptococcus dysgalactiae* subspecies *dysgalactiae*, EndoE from *Enterococcus faecalis*, or *Streptomyces griseus*, have also been identified as enzymes with specific antibody modulating activities distinctive from EndoS and EndoS2 [155,163,165,166,169,170]. Besides their contribution to GAS pathogenicity, several of these enzymes are used as, or are being developed into, therapeutic agents or biotechnological tools for antibody modification, analysis or purification. EndoS and EndoS2 and their potential as biotechnological tools will be described in more detail in chapter 4.

The development of a GAS vaccine

One of the most important scientific contributions to human life-expectancy and health was the recognition that people who survived infections acquired immunity against subsequent infections, which eventually led to the development of the first successful vaccine against smallpox (1796), followed by vaccines against rabies, typhoid, cholera, polio etc. [171,172]. As mentioned in the previous chapter, *Streptococcus pyogenes* is a ubiquitous human pathogen causing a spectrum of different diseases, leading to suffering for the individual, and a considerable burden on the health care systems world-wide. This has created a need for better control and prevention measures for *S. pyogenes infections*, and has been a great incentive for a vaccine development during the last decades. The fact that there are no licensed vaccines for *S. pyogenes* is quite remarkable considering the long history of *S. pyogenes* research and the availability of detailed knowledge on molecular pathogenesis, the structure and function of numerous virulence factors and the protective immune response in various animal models of infection. The development of an efficient vaccine against GAS has been difficult and delayed by several factors, including the large strain diversity, antigenic variation and geographic differences in serotype distribution in prevalence and diseases. There are a number of promising *S. pyogenes* vaccine targets, including carbohydrate and fibronectin binding proteins, streptolysin S (SLS) and streptolysin O (SLO), however the main target has long been those based on the M-protein which is seen as a main virulence determinant of the organism. More recently a polyvalent vaccine candidate was developed, a recombinant four-epitope protein, showing effective protection against infection with several *emm* types of *S. pyogenes* in mice [173–175]. Another major concern has been the fact that *S. pyogenes* antigens are known to contain autoimmune epitopes, mainly found in cardiac tissues, which can trigger ARF and lead to rheumatic heart disease (RHD) [174,176]. In the context of this thesis, considering that the underlying immunological mechanism of vaccination is based on the induction of protective antibody mediated immunity, it becomes interesting to question whether *S. pyogenes* antibody modulating enzymes play a role, and should possibly be considered, in vaccine development. The development of protective immunity against *S. pyogenes* is generally poor, and although type-specific antibodies are generated following a natural infection, no clear correlation has been demonstrated between the persistence of such antibodies and the severity of pharyngeal infection [177–179]. Studies have shown that there is a natural development of antibodies towards both IdeS (anti-IdeS) and EndoS (anti-EndoS) following *S. pyogenes* infection or following repeated administration of the enzymes in different animal models of antibody mediated autoimmune disease. Whilst the generation of specific anti-IdeS antibodies has been shown to neutralize IdeS activity, resulting in decreases survival of *S. pyogenes*, the generation of anti-EndoS

antibodies has not been shown to affect the enzymatic activity of EndoS [180–182]. However, conflicting results from another study demonstrated that vaccination using EndoSe (displaying approximately 70 % identity with several EndoS sequenses) resulted in a significant protection against *Streptococcus equi* subsp. *equi* infection in horses [168]. These conflicting results could potentially be explained by the quality, i.e. their neutralizing capacity, of the generated antibodies, as well as the physiological enzyme concentration, which is certain to differ substantially between an animal injected with a recombinant enzyme as compared to the barely detectable amounts produced during a bacterial infection.

Alas, the work towards an effective vaccine to protect against *S. pyogenes* infections continues, and the role of the bacterial antibody modulation in this search remains unclear. However, the activity of these enzymes should not be ignored, as it has been shown that these contribute to immune evasion during infection [167,168].

Bdellovibrio bacteriovorus

Predatory bacteria, and *Bdellovibrio bacteriovorus* in particular, have had a recent surge in attention. Much of the interest is with regard to the use of predatory bacteria as biological-based agents, also referred to as live antibiotics, to treat multi-drug resistant (MDR) bacterial infections [183,184]. However, as will be further described in this chapter, the application of predatory bacteria is more versatile, especially focusing on their plethora of hydrolytic enzymes and their potential as sources for the discovery of novel biotechnological tools.

The existence of predatory bacteria has been long known, and since the first documented study in the 1950s many new predatory bacterial strains, predation strategies and potential applications have been described. One of the best studied predatory bacteria is *Bdellovibrio bacteriovorus*, a small, highly motile, Gram-negative δ-proteobacteria, known to employ an endobiotic hunting strategy which entails invading, and proliferating within the periplasm (between the inner and outer membrane) of Gram-negative bacteria, including several known human pathogens like enterohemorrhagic *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas* and *Salmonella* [185–187]. *Bdellovibrio* employs a biphasic lifestyle, with a free swimming “attack phase” and an intraperiplasmic growth and replication phase. In the free-swimming phase, *Bdellovibrio* randomly collides with, and attaches to Gram-negative prey cells, invading by creating a pore in the outer membrane, crossing the peptidoglycan layer, and establishing itself within the prey periplasm. Upon collision *Bdellovibrio* has been reported to remain reversibly attached for a short “recognition” period before becoming irreversibly anchored [188–190]. It has been suggested that *Bdellovibrio* utilizes its type IV pili to pass through membrane,

shedding the flagellum and resealing the pore following entry. Upon entry into the periplasm *Bdellovibrio* forms the bdelloplast, an osmotically stable niche, in which it consumes prey macromolecules as nutrients, growing filamentously without any competition. Bdelloplast formation causes a distinct rounding up of the usually rod-shaped prey cell resulting from peptidoglycan cell wall modifications. This modification has been shown to promote 1:1 predator to prey ratio, preventing self-competition between individual predators for the same prey. When the prey is exhausted, the filamentous predator septates into flagellated progeny cells, followed by host cell lysis and progeny release. Whereupon the cycle begins anew (Fig. 6) [189–192].

Bdellovibrio are found in a wide variety of environments including soil and different aquatic habitats. *Bdellovibrio* has occasionally also been found in the mammalian gastrointestinal tract, without causing disease, rather it has been associated with health [193–195]. The potential use of live bacteria as therapeutics naturally raises questions and concerns regarding efficacy and safety of *Bdellovibrio* administration. This has been thoroughly investigated in several animal models, as well as with human cells, showing the inability of *Bdellovibrio* to invade mammalian cells, and displaying no signs of cytotoxicity or reduction in cell viability, supporting the proposition that *Bdellovibrio* is inherently non-pathogenic to mammals [196–198]. *Bdellovibrio* is primarily recognized as an obligate predatory bacterium, requiring living prey bacteria for survival. Interestingly, *Bdellovibrio* can switch to a host-independent (HI) lifestyle, displaying either saprophytic (requiring prey extracts, also referred to as Type I mutants) or axenic (growing on complete media without prey components, also referred to as Type II mutants) growth. The initial events leading to HI growth are still obscure, however studies investigating changes in gene expression between HI- and obligate predatory *Bdellovibrio* have shown distinct up- and down regulations of specific genes. Such differences include downregulation of genes associated with prey location and initial interaction in HI *Bdellovibrio* and an up regulation of predation-specific genes in host dependent (HD) *Bdellovibrio* [199–202].

The predatory lifestyle of *Bdellovibrio* requires a plethora of hydrolytic enzymes, utilized during prey cell entry, degradation of prey biopolymers and the final lysis of the bdelloplast during release of the progeny cells. The *Bdellovibrio* genome encodes 150 annotated proteases and peptidases, 10 glycanases, 20 DNAses, 9 RNAses, and 15 lipases, as compared to 83 annotated hydrolases in *S. pyogenes* (www.uniprot.org September 2019) [189]. This hydrolytic arsenal also makes *Bdellovibrio* an interesting source for identifying novel bacterial proteins with applications within basic research and the life science industry. This potential can be exemplified by the identification and characterization of BspK (*Bdellovibrio* serine protease K) and BspE (*Bdellovibrio* elastase-like serine protease) with described enzymatic activities on human antibodies. The identification of novel

antibody-degrading or -modifying proteins is, due to the rapid development and increasing number of approved mAbs on the market, greatly needed.

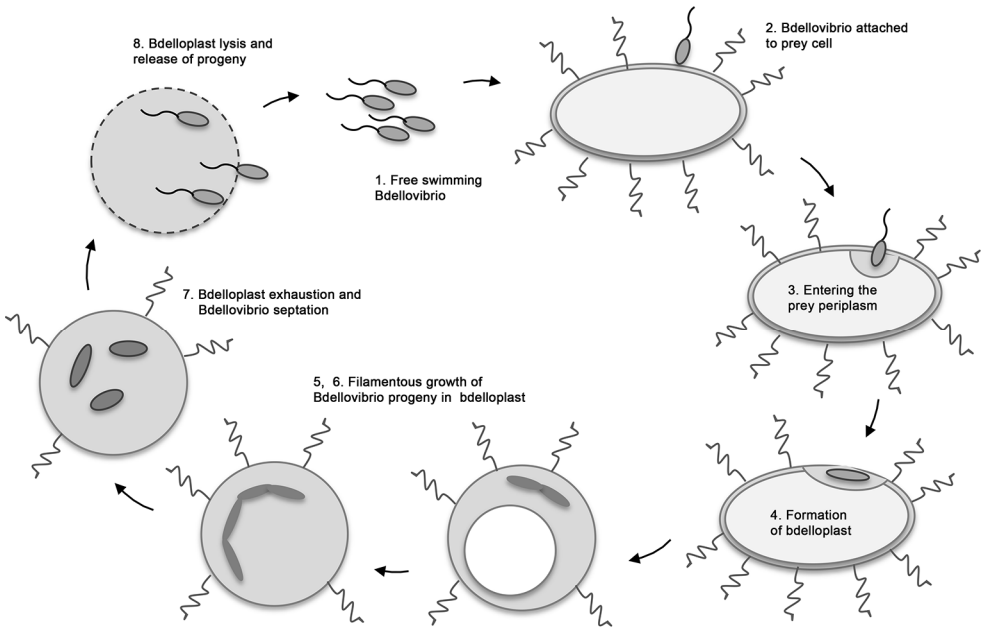

Fig. 6.
Representation showing the biphasic life cycle of *Bdellovibrio bacteriovorus*. 1. Free-swimming *Bdellovibrio* collide randomly with prey cell, followed by reversible and subsequently by irreversible attachment 2. to prey. *Bdellovibrio* generates a pore in the prey outer membrane and 3. enters the prey periplasm (crossing the peptidoglycan layer) where it forms the bdelloplast 4. Within the bdelloplast, *Bdellovibrio* consumes prey macromolecules as nutrients, growing filamentously, exhausting the prey 5-7. Finally, the filamentous predator divides into flagellated progeny cells, followed by host cell lysis and progeny release.The general shift from the classical chemical drugs towards biopharmaceuticals, and mAbs in particular, requires reproducible and reliable analysis and quality control. These analyses are commonly performed by mass spectrometry-based methods, requiring fragmentation of intact proteins into smaller peptides, emphasizing the need for a larger selection of biotechnological tools for this purpose [203–205]. This creates a need for hydrolytic enzymes displaying unique and complementary cleavage profiles for antibody analysis, making *Bdellovibrio* an interesting candidate for the identification of new enzymes with biotechnological applications. The figure is modified from Dwidar M, Monappa AK, Mitchell RJ. The dual probiotic and antibiotic nature of *Bdellovibriobacteriovorus.* BMB Rep. 2012 Feb;45(2):71-8.

4. Therapeutic antibodies and tools for modification

Much has happened since the generation of the first mAb nearly half a century ago, establishing the field of therapeutic and diagnostic antibodies. Continuous research has resulted in an immense accumulation of knowledge, which has contributed to the development of therapeutic mAbs with improved safety, clinical efficacy and precision. This scientific progression has also contributed greatly to the ongoing switch, from classical chemically based drugs to biopharmaceuticals, on the pharmaceutical market where mAbs, Fc-fusion proteins, Ab fragments and Ab-drug conjugates (collectively referred to as mAb therapeutics) currently represent the dominant group of therapeutic products on the market [206,207]. The importance of the Fc N-glycan for IgG effector functions, and the understanding of how distinct glycoforms influence the elicited immune response, has led to the development of various glycoengineering strategies applied for the development of improved mAb-therapies. Importantly, non-human glycans can potentially be immunogenic, emphasizing the importance of rigorous and reliable quality controls and analysis methods [48,208]. This chapter will highlight the development and applications of therapeutic mAbs, mAb glycosylation, glycoengineering and quality control of antibodies for basic research and therapeutic use.

The discovery of mAb technology dates back to 1975 and laid the foundation for using antibodies as therapeutics. However, it wasn't until 1986 that the first mAb for therapeutic application was approved by the FDA (U.S Food and Drug Administration). Orthoclone OKT3, a mouse mAb specific towards the CD3 epitope on human T-cells, was applied for the treatment of T-cell mediated graft rejection following kidney transplants. Naturally, the administration of a mouse derived protein caused complications with repeated treatments, as the inherent immune response results in the generation of antibodies towards non-self-substances, which limited the applicability of the drug. To address the issues of immunogenicity genetic engineering was applied to create chimeric mAbs, combining mouse V-regions with human C-regions, reducing the risks of complications following subsequent treatments. Rituximab, a chimeric mAb specific for the CD20 cell surface protein on B-cells, has since its approval use in 1997 been used to treat over one million patients [209]. In late 2018 there were > 80 mAbs that had been

approved for marketing, with over 570 antibody therapeutics currently being evaluated at various clinical phases, clearly demonstrating the development, efficiency and broad applicability of antibody therapeutics [210,211].

Glycosylation of therapeutic antibodies

The mAb market is dominated by human or humanized antibodies of the IgG1 subclass, and to some extent IgG2 and IgG4. The heterogenic nature of antibody production (see previous chapter on antibody structure and diversity) results in a variety of modifications giving rise to a large diversity (10^9 -10^{12}) in an individual antibody pool. Naturally, this diversification also applies to the recombinantly expressed therapeutic mAb, and could have tremendous implications for the clinical efficacy and safety of an antibody-based drug. The importance of glycosylation for structural, functional and biological properties of antibodies in general is described in previous chapters of this thesis.

Glycosylation is, as previously described a dynamic and complex process taking place in the ER and Golgi, and this PTM influences antibody properties including protein structure, stability, half-life, solubility, secretion, immunogenicity and antibody mediated effector functions. Thus, keeping control of the glycosylation and knowing the glycosylation profile is essential to ensure the safety and clinical efficacy of any therapeutic mAb or other Ab-based products including Fc-fusion proteins, Ab-drug conjugates and antibody-enzyme fusions. All therapeutic IgG's are modified by the conserved Fc *N*-linked glycan, and some also carry additional Fab glycans e.g. Cetuximab (a chimeric mouse/ human mAb, inhibiting the epidermal growth factor receptor (EGFR), used for targeted therapy of colorectal, head and neck cancers) [212,213]. It is well known that the mAb Fc glycoform directs the elicited antibody mediated effector functions, however the function of mAb glycosylation in the variable Fab domain remains to be determined. Importantly, the glycosylation is entirely dependent on the nature of the expression system (cell line) used for mAb production.

A consistent mAb production (essential for therapeutic applications) also requires a well-established cellular production system, including the bioreactor environment. Slight changes in cell culture conditions, including dissolved oxygen levels, nutrient levels, temperature, osmolality and pH, can considerably alter the glycan profiles of the produced mAbs. There are several different systems that can be used for the production of human glycoproteins, including yeast, plant and insect cells, however without any bioengineering only mammalian cell lines are capable of producing human-like complex glycans. Although the development and use of therapeutic mAb is becoming increasingly common there are still problems with the present

expression systems used for therapeutic glycoprotein production. An important issue is the general struggle to produce glycoprotein modified with uniform glycan structures. Another issue concerns the current cell lines primarily used for mAb production. Although there is an ongoing shift towards the use of human cell lines for mAb production, including the use of HEK293 (human embryonic kidney 293), fibrosarcoma HT-1080 and PER.C6 cells, the predominantly used systems currently being used are based on CHO (Chinese hamster ovary) cells (approx. 70% of all currently approved therapeutic mAbs [214]), NS0 and Sp2/0 (murine myeloma cells) cell lines. This is a problem as these non-human cell lines, although capable of producing human-like complex glycans, also generate non-human glycan structures that can reduce mAb half-life and adversely affect safety, causing unwanted side effects [214–217].

Cetuximab is a good example, also emphasizing the importance of quality control and the importance of correct mAb glycosylation. This chimeric mouse/ human mAb is produced in the Sp2/0 mouse myeloma cell line known to produce glycoforms containing *N*-glycolylneuraminic acid (NGNA) and galactose-α-1,3-galactose (Gal-α-1,3-Gal) structures. These glycoforms are nonexistent in humans making them potentially immunogenic. A comprehensive analysis of Cetuximab glycosylation showed two N-glycosylation sites including the conserved site in the Fc domain, and a second site in the Fab region. The results demonstrated that Cetuximab is modified with a complex mixture of different glycans, identifying 21 different glycoforms. Approximately 30% of the glycans were capped by at least one Gal-α-1,3-Gal- and 12% by an NGNA- residue. Importantly, these glycoforms were only present in the Fab region [212,218–220]. The safety and efficacy of Cetuximab has been tested and proven to be excellent in several clinical trials, and it has been approved for treatment in 40 countries. Several thousand patients have successfully been treated, however there have also been reports on cetuximab-induced anaphylaxis where preexisting, galactose-α-1,3-galactose specific, IgE antibodies were detected in patients treated with the mAb [221]. The development of new technologies including phage display and genetically humanized animals, usually mice or rats, has enabled the generation of fully human antibodies lacking any murine sequences. These transgenic – humanized – animals are genetically modified through the introduction of human immunoglobulin loci while knocking out their inherent antibody genes. Accordingly, immunization leads to the generation of antibodies from human germ-line sequences, allowing subsequent generation of mAbs by classical hybridoma technology. The development of antibody phage display has also contributed greatly to the generation of specific mAb without immunization [222–225]. Together with the ongoing evaluations, and engineering of human cell lines for optimal mAb production and yield, chances are that there will be a future switch towards a use of fully humanized therapeutic mAb production systems.

Structural changes, including glycoform heterogeneity as well as natural processing and degradation events taking place during production, can led to product variants of the therapeutic glycoproteins. Such variations can lead to altered properties, including activity (receptor interactions), clearance rate, serum half-life, immunogenicity, stability, solubility and tendency to form immune complexes/ aggregates of the drug [220,226–228]. By now, it might already be evident why a comprehensive analysis of the biotherapeutic molecule, including the glycosylation, is essential. The influence of mAb glycosylation on efficacy and safety makes the analytical characterization of glycosylation forms an essential part of protein structure analysis throughout the development and production process. The significant impact of IgG Fc-glycosylation on antibody mediated effector functions, including ADCC, CDC and ADCP makes it of particular importance to know the glycan profile of the mAb, as one of these effector functions might be the primary therapeutic mechanism of the drug.

Despite the analytical improvements made during the last decade, the characterization of mAb heterogeneity and PTM during production remains a challenge. The main characterization methods currently applied are liquid chromatography (LC) and mass spectrometry (MS), most often used in combination (LC-MS). Characterization of protein modifications by LC-MS most commonly requires proteolytic degradation of the protein into smaller peptides prior to analysis, referred to as bottom-up (BU) analysis. BU analysis provides high site-specificity, however the digestion into small peptides can complicate the analysis due to the generation of artifacts during lengthy digestions. Additional drawbacks to peptide analysis are complicated sample preparation, lengthy analysis and time-consuming data processing that prevents this method from being used for routine monitoring of product quality during production [229,230]. The development of top-down (TD) and middle-down (MD) MS approaches, and the combination of both, has enabled thorough characterization of mAbs, yielding an overall sequence coverage and PTM mapping, which is at least equivalent to BU analysis. TD and MD MS refers to the analysis of intact proteins or proteins cleaved into protein subunits, respectively [231,232]. Sample preparation for MD and TD analysis also reduces the likelihood of sample manipulation, limiting the risk of artefactual findings during analysis [229,230,233].

The difficulties of generating uniformly modified mAbs with the desired glycan structure, with the currently available production systems, emphasizes the need for adequate and reliable methods for mAb quality control, as well as the need for improved methods for mAb production.

Monoclonal antibody engineering

The accumulation of knowledge and rapid development of novel therapeutic mAbs, together with the increased demand of therapeutic mAbs on the market has resulted in efforts being made to address the issues of inconstant PTM and hinge susceptibility for proteolytic cleavage. Several strategies have been developed to improve the yields and stability, in regards to end product quality and uniformity, of the currently used mAb production systems. These strategies are mainly based on protein- and glycoengineering and include site directed mutations (SDM) in the primary protein sequence, the deletion of specific genes involved in glycan synthesis, or the overexpression of specific glycosyltransferases in the presence of the corresponding substrate nucleotide sugars. Other glycoengineering approaches have focused on enzymatically modifying the end product.

Antibody glycoengineering

All the accumulated knowledge related to antibody structure and function, and the functional importance of glycosylation has been fundamental for the development of antibody-based therapeutics. To overcome the issue of unwanted glycan structures and heterogenic glycosylation, different glycoengineering strategies have been developed to achieve a uniform production of the desired mAb glycoform.

Numerous studies have shown that the removal of the core fucose bound to the innermost GlcNAc residue on the IgG Fc N-glycan results in an increased affinity and binding to FcγRIIIa, leading to recruitment of mononuclear cells (primarily NK-cells) and enhanced ADCC, as compared to fucosylated antibodies. This knowledge has been applied to develop glycoengineering strategies to reduce core fucosylation on therapeutic mAbs. One of the main strategies involves knocking out the α1,6-fucosyltransferase8 (Fut8) gene in the production cell line, preventing the addition of the core fucose to the conserved complex N-glycan on IgG [227,234,235]. Another strategy to reduce core fucosylation has been to overexpress β1,4-N-acetylglucosaminyltransferase (GnT-III) increasing the production of antibodies with bisecting GlcNAc residues and therefore outcompeting the fucosyltransferase catalyzing the addition of the core fucose. The exact mechanisms underlying this inverse correlation is still not fully understood, however it is speculated that addition of a bisecting GlcNAc results in steric hindrance obstructing the addition of a core fucose. Knocking out the genes involved in the GDP-fucose (donor substrate for fucosylation) biosynthesis is an alternative strategy to reduce core fucosylation. However, this strategy eliminates overall fucosylation and is not limited to the α1,6-fucosyltransferase8 (Fut8) addition of the core fucose [47,234].

Sialylation of the IgG Fc N-glycan is in many instances a highly desirable feature for a therapeutic mAb. Terminal sialic acids mask the galactose residues, preventing interaction with the asialoglycoprotein receptors on hepatocytes resulting in the increased mAb half-life. Research has also shown increased IgG Fc sialylation enhances the anti-inflammatory properties of the antibodies. Terminal galactosylation has in turn been associated with increased binding to C1q resulting in enhanced CDC. Thus, the glycosylation profile needs to be closely considered and engineered accordingly, depending on the desired therapeutic mechanism of the mAb. Glycoengineering strategies for enhanced sialylation and galactosylation includes overexpressing the α2,6-sialyltransferase and β1,4-galactosyltransferase, as well as genetic engineering of the production cell line by knocking out genes encoding specific sialyltransferases, that compete with the α2,6-sialyltransferase, generating unwanted sialylated glycoforms [88,90,234,236–238].

Although the above-mentioned antibody engineering strategies have contributed to an improved production of mAbs uniformly modified with the desired glycan structures, the optimization of the production process is still challenging. As an alternative there is now a recently developed glycoengineering strategy using transglycosylation reactions and modifications to remodel the pre-existing glycan structure on the end product. This is achieved by a multi-step process starting with the deglycosylation of the antibody by using bacterial ENGases (endo-β-acetylglucosaminidases), leaving only the innermost GlcNAc residue with or without an attached fucose residue. This is followed by a subsequent reglycosylation, achieved by transferring intact, predefined glycan substrate to the innermost GlcNAc residue by using an ENGase-based glycosynthase mutant (endoglycosidases modified to favor the reversed reglycosylation reaction). These ENGases include EndoS and EndoS2 (*Streptococcus pyogenes)* and many more. The different ENGases display distinct enzymatic properties with individual substrate specificities and limitations. EndoS specifically removes complex-type N-glycans from the IgG Fc, whilst EndoS2 also cleaves high mannose- and hybrid-type glycan structures [164,239–241]. This method of glycoengineering of the mAb end-product, allows for a controlled, uniform production of antibodies modified with the desired, pre-defined glycan structure.

These described methods of IgG Fc engineering are contributing to the development of new and improved antibody-based drugs, providing strategies to optimize efficacy, safety and functionality. The optimization of mAb production will hopefully also contribute to making these biological drugs more affordable and thus more available in the coming decade.

Engineering of the antibody hinge region

The susceptibility of the IgG hinge region to proteolysis is a well-known issue, as cleavage of the hinge renders the therapeutic mAb or Fc-fusion proteins non-functioning. Proteolysis of the hinge can occur through physiological processes, including radical reactions during antibody production, but also through the action of a number of host and bacterial proteases. Cleaved IgG has been found *in* vivo in a number of pathological settings including different forms of cancer, cystic fibrosis, arthritis and sepsis. Single hinge cleavage, in one of the antibody HC, has shown to impair mAb mediated effector functions [167,242–245]. There are several strategies to reduce mAb degradation during cell culture including optimizing the timing of the harvest (to limit exposure to the proteases), reducing temperature (lowering enzymatic activity), optimizing pH, continuously adding protease inhibitors or substitute substrates. Another strategy to address this issue has been engineering of the antibody hinge region by directed point mutations, substituting specific amino acids in the proteolytic site to protect the mAb from fragmentation [243,244,246–248].

The integrity of the hinge region is essential for antibody structure, flexibility and function and any alteration to the primary protein sequence may affect mAb safety and clinical efficacy. Thus, the outcome of any modification needs to be carefully considered and thoroughly evaluated. Recent studies have shown that a specific site directed substitution in the IgG1 hinge improved the stability and function of the antibody, whilst it still retained a similar pharmacokinetic profile and a 2-3-fold increase binding to FcγRIII compared to the native molecule enhancing the mAb ADCC activity [249].

Antibody binding and purification

Another critical step in the production of antibodies is purification following expression. The established purification process needs to be reliable and provide products suitable for their down-stream application, which for mAbs requires suitability for administration to patients. The current strategies used for mAb recovery are primarily based on affinity chromatography (AC), using classical immunoglobulin binding proteins (IBPs) including protein G, protein A (predominantly used) and protein L, in combination with centrifugation, and different filtration methods. It should be acknowledged that the use of protein A for capture of the antibody and removal of host cell impurities (Protein, DNA) is extremely efficient. However, the high cost of protein A resins, the harsh elution conditions using low pH buffers and the lack of discrimination between intact and correctly folded or defective antibodies are critical shortcomings that should be

recognized. Furthermore, potential leaching of these IgG binding proteins, originally identified from Group G streptococci (protein G), *Staphylococcus aureus* (protein A) and *Peptostreptococcus magnus* (protein L), into the purified mAb batch is another potential issue that needs to be considered [250–253]. Protein A and protein G bind specifically to IgG, interacting mainly with the protein structure of the Fc-region. Considering the importance of Fc N-glycosylation for IgG mediated effector functions the complete disregard of the glycan structure is yet another limitation when using these traditional purification platforms. The harsh elution conditions are a concern as it has been linked to antibody aggregation and the low pH also risks damaging the purified antibodies, rendering them useless in many downstream applications. Other purification methods based on ion exchange or hydrophobic interactions are less selective to the antibody, reducing the purity in a one-step process, as compared to the IgG specific AC.

A more recent alternative to the traditional IBPs is the antibody-based affinity resin termed CaptureSelect™, based on HC antibodies (lacking the entire LC) naturally produced in camels and llamas. These antibodies have a larger hypervariable region and display a broad antigen diversity, interacting with their antigen through their single variable domain (VHH to distinguish it from VH). CaptureSelect provides great selectivity to their target antigen which enables purification from crude cell culture media or complex materials such as blood. However, the issues of harsh elution conditions and the disregard of the antibody quality and glycan modifications remain, emphasizing the need for improved antibody purification methods [254–256].

The above described issues and limitations associated with the use of the traditional IBPs and CaptureSelect, we are currently evaluating a novel method for selective IgG-purification using enzymatic inactive variants of bacterial IgG-specific glycan hydrolases (further described in chapter 5, *Paper IV*).

5. Present investigation

Paper I

The recent advancement of biological molecules as therapeutic agents, and especially the rapid development of therapeutic monoclonal antibodies, has in parallel created a need for novel tools and innovative methods to ensure their quality and safety. The application of bacterial enzymes as biotechnological tools for antibody analysis is already well established, thus we propose the predatory bacterium *Bdellovibrio bacteriovorus* with its plethora of hydrolytic enzymes to be an excellent source for the identification of novel enzymes with biotechnological potential. Here we identified and characterized BspK (*Bdellovibrio* serine protease K), a novel serine protease from *B. bacteriovorus,* describing its hydrolytic activity on antibodies. We identified a preferential hydrolysis of human IgG1 compared to other immunoglobulins and isotypes, hydrolyzing the antibody in the HC generating two separate Fab fragments and an intact IgG Fc domain. We further describe the enzymatic characteristics of BspK, demonstrating a unique cleavage profile compared to several currently used proteases on the market.

Paper II

During the work on *Paper I* we found evidence of other proteases active on IgA stressing the diversity of enzymes in *Bdellovibrio.* Here we identified and characterized the novel protease BspE (*Bdellovibrio* elastase-like serine protease) from the predatory bacterium *Bdellovibrio bacteriovorus*, demonstrating a single proteolytic cleavage site in the Fc-tail of native plasma IgA (pIgA); as well as in the secretory component (SC) of secretory IgA (SIgA). The hydrolytic activity on pIgA was subsequently utilized for the specific glycan characterization of the N- linked glycan (Asn^{459}) on the pIgA Fc-tail. These findings contribute to the basic knowledge of *Bdellovibrio* and could potentially facilitate future investigations looking into the importance, and biological function of the pIgA Fc-tail.

Paper III

The bacterial enzyme Endoglycosidase S (EndoS) secreted by *Streptococcus pyogenes* is well known for its specific activity, cleaving the conserved N- glycan attached to Asn-297 in the CH2 domain of IgG. The molecular and biochemical mechanisms underlying this enzymatic activity, and substrate specificity are well

understood. EndoS activity has also shown to impair IgG mediated effector functions *in vivo.* However, the relevance of EndoS activity to GAS infection has remained unclear. Here we addressed this question by characterizing the effects of EndoS on host IgG glycosylation during ongoing infections by analyzing samples from patients with sepsis. In addition, we established a model of invasive *S. pyogenes* infection in naïve and immunized mice. The results demonstrated a substantial hydrolysis of the conserved IgG glycan at the site of infection, and this could also be detected systemically in the most severe cases. Removal of EndoS resulted in decreased survival of GAS in vitro and reduced virulence in a mouse model of invasive infection. This study supports the hypothesis that EndoS modifies antibodies in vivo and thus contributes to bacterial virulence.

Paper IV

The molecular mechanism underlying the substrate specificities of EndoS and EndoS2 has recently been described in detail, showing direct interactions between the enzyme and the glycan. Here we evaluate the potential of using enzymatically inactive, immobilized $EndoS_{E235L}$ and $EndoS2_{E186L}$ for antibody purification based on these specific protein-glycan interactions, potentially enabling a selective purification based on IgG Fc N-glycan structure. We show that $EndoS_{E235L}$ and $EndoS2_{E186L}$ can be used to selectively purify IgG from a complex sample such as human serum. The results also show that a removal of the Fc N-glycans using EndoS or PNGaseF, prior to the affinity purification results in a significant but not a complete loss in IgG binding. These results confirm that much of the interaction between the immobilized inactive enzyme and IgG relies on interactions with the N-glycan. We also address the problem of harsh elution conditions required when using traditional methods of antibody purification by establishing gentle coupling and elution conditions, an important aspect as this enables a selective purification of native, glycosylated and otherwise undamaged antibodies.

Discussion and future directions

Nearly a century has passed since the discovery of antibodies, and the accumulation of knowledge, including details on structure, function, PTMs and receptor interactions, has contributed to our current understanding of their diverse function in the immune system. The work in this thesis presents different aspects of bacterial antibody modulation, showing how this strategy is used by bacteria to evade host immunity and how these bacterial enzymes are applied as tools in research and industry to develop new technologies and biopharmaceuticals.

It is well known that *Streptococcus pyogenes* produces several antibody modifying enzymes that are considered to be involved in evasion from host defences. In our work we show, for the first time, that EndoS is expressed and active *in vivo* during an infection with *S. pyogenes*. We could clearly detect EndoS hydrolysis of the IgG Fc N-glycan, rendering the antibodies incapable of mediating the effector functions crucial for resolving the bacterial infection, clearly showing that EndoS can contribute to *S. pyogenes* pathogenicity. By establishing an animal model of invasive *S. pyogenes* infection in mice we investigated the role of EndoS in both naïve and immune conditions (pre-immunizing mice using the bacterial M-protein). The contribution of EndoS to bacterial survival was only evident in the context of adaptive immunity, indicating that innate immunity plays a minor role in the protection against the pathogen and that EndoS is capable of neutralizing the protective pre-existing antibodies in circulation, crippling the adaptive response towards the pathogen. The effective neutralization of IgG by *S. pyogenes* offers a possible explanation as to why the protection with pre-existing antibodies towards the bacterium is generally poor.

Our results also showed there is great variation in EndoS expression levels between different *S. pyogenes* isolates, but there was no clear association with CovRS mutation or specific *emm* type. This suggests that EndoS expression is not exclusively regulated by CovRS, supporting previous reports showing the involvement of the transcriptional regulator CcpA for EndoS expression. Importantly, this indicates that EndoS expression is not restricted, but rather generally expressed by many different *S. pyogenes* serotypes. This is further supported by the conservation of the *ndoS* gene throughout the *S. pyogenes* serotypes and the presence of numerous EndoS homologs in other streptococcal strains including *Enterococcus faecalis, Streptococcus pneumonia* and

Streptococcus dysgalactiae. Taken together with the results from another study showing that an EndoS homolog from *S. equi* conferred protective immunity in a vaccination trial in mice, suggests that EndoS, and the action of other IgG degrading enzymes, should be considered in the development of a *S. pyogenes* vaccine.

The development of antibody-based therapeutics that has been ongoing during the recent decades and the understanding the importance of IgG Fc N-glycosylation, and how modifications to the glycan structure can tune the elicited immune response, has added another layer to the development these therapeutics. This knowledge is now allowing the design of mAbs with enhanced clinical efficacy and targeting of the mAb therapeutic mechanism of action towards enhanced ADCC, CDC or ADCP by specifically modifying the primary protein sequence or the glycosylation of the antibody. The rapid development of antibody-based therapeutics has also created a need for new methods and tools for product development, for the subsequent analysis to ensure quality and safety, and for improved antibody purification. Interestingly, many of these methods are enzyme-based, utilizing bacteria derived enzymes with specific activities on antibodies, e.g. transglycosylation of mAbs using glycosynthase mutants, used to produce antibodies modified with the desired and uniform glycan structures. The identification and characterization of BspK and BspE presented in this thesis are two examples demonstrating the potential of using the predatory bacterium *Bdellovibrio bacteriovorus* as a source for the identification of novel enzymes for the use as biotechnological tools for research within both academia and industry. The large amount of annotated hydrolytic enzymes in the *B. bacteriovorus* genome makes it an interesting candidate for the discovery of novel enzymes with biotechnological applications.

This thesis shows the importance of basic research, how the detailed knowledge of a molecular structure, or the understanding of a molecular mechanism and biological process, is essential for the development of new therapeutic products and technologies.

Concluding remarks

In this thesis the importance of antibody glycosylation for antibody mediated effector functions in adaptive immunity, as well as for the clinical efficacy of therapeutic antibody-based drugs becomes evident.

This work presents several aspects and applications of antibody glycan modifications, demonstrating for the first time how deglycosylation of IgG *in vivo*, during invasive infection with *Streptococcus pyogenes*, contributes to bacterial pathogenicity in the context of adaptive immunity. This work also presents the potential of using predatory bacteria as sources to discover new bacterial enzymes with biotechnological applications. And how bacterially derived antibody modulating enzymes can be used for different biotechnological applications including; antibody analysis and quality control during the antibody production process; specific glycoengineering of therapeutic mAbs, resulting in better production yields and product uniformity as well as improved targeting and increased clinical efficacy; and potentially as binding reagents for selective antibody purification.

Sammanfattning

Vårt immunförsvar består av ett nätverk av olika organ, celler och proteiner som skyddar kroppen mot inkräktande sjukdomsframkallande mikroorganismer och andra främmande ämnen. Immunförsvaret är ofta uppdelat i två grenar, det nativa, även kallat det medfödda, försvaret som känner igen viktiga ytstrukturer på olika bakterier eller virus, och det specifika adaptiva försvaret som bidrar till ett specifikt, bestående skydd mot återkommande infektioner. Antikroppar utgör en mycket viktig del av det adaptiva immunförsvaret. Dessa proteiner är specifika och känner endast igen en eller ett fåtal struktur(er) till vilka de binder. Antikropparna används av kroppen för att upptäcka och identifiera främmande ämnen och mikroorganismer, för att sedan aktivera immunförsvaret och eliminera faran. Antikroppen IgG (Immunoglobulin G) är den mest förekommande antikroppen i blodet, och är ett av de mest välstuderade glykoproteinerna (protein modifierat med sockerkedjor som bidrar till struktur och funktion). Tidigare forskning har påvisat att de konserverade sockerkedjorna på IgG är nödvändiga för antikroppens fulla funktion.

Streptococcus pyogenes är en sjukdomsframkallande bakterie som orsakar både vanligt förekommande infektioner som halsfluss, men också livshotande infektioner i mjukvävnad och sepsis. *S. pyogenes* har under samevolution med människor utvecklat många strategier för att undvika immunförsvaret, däribland enzymet Endoglycosidase S (EndoS). EndoS klyver specifikt sockerkedjorna på IgG, vilket medför att antikroppen förlorar sin kapacitet att aktivera immunförsvaret. Ur ett rent biologiskt och evolutionärt perspektiv är förmågan att inaktivera det antikroppsmedierade immunförsvaret en mycket fördelaktig bakteriell egenskap, då det skulle kunna underlätta ett infektionsförlopp.

Den snabba utvecklingen av nya antikroppsbaserade läkemedel, samt den breda användningen av antikroppar inom akademisk och industriell forskning, men även för medicinska analysmetoder, har skapat ett behov av nya bioteknologiska verktyg för vidareutveckling och kvalitetskontroll av kommersiella antikroppar. Majoriteten av dagens analysmetoder för antikroppar är enzymbaserade och kräver att antikroppen klyvs ner i mindre bitar och många av enzymerna som används i detta syfte kommer ursprungligen från bakterier. *Bdellovibrio bacteriovorus* är en predatorisk bakterie som jagar, invaderar och utnyttja andra bakterier för att överleva. Den predatoriska livsstilen kräver en stor mängd enzymer som används i olika faser under livscykeln, och identifieringen av två nya enzymer med aktivitet

på mänskliga antikroppar från den predatoriska bakterien *Bdellovibrio,* belyser potentialen av att använda denna bakterie för att upptäcka fler nya bioteknologiska verktyg.

I artikel I identifierade och karaktäriserade vi ett tidigare okänt enzym med enzymatisk aktivitet på olika antikroppar, från den predatoriska bakterien *Bdellovibrio bacteriovorus.* Genom olika molekylärbiologiska metoder kunde vi identifiera rätt protein, varpå vi producerade och renade det för att sedan undersöka den enzymatiska aktiviteten på antikroppar i detalj. Det nya enzymet BspK (*Bdellovibrio* serine protease K) visade sig ha mer eller mindre specifik aktivitet på ett antal olika antikroppar från människa, men även andra djur. Av störst intresse var den specifika BspK aktiviteten på IgG1 som delar antikroppen i tre distinkta bitar. Våra resultat bidrar till den basala kunskapen om *Bdellovibrio* och antyder att BspK skulle kunna ha potential för att utvecklas till ett nytt bioteknologiskt verktyg för olika typer av antikroppsanalys.

I artikel II upptäckte vi att *Bdellovibrio* har en annan intressant enzymatisk aktivitet på antikroppen immunoglobulin A (IgA). Målsättningen med arbetet var att identifiera och karaktärisera ett sedan tidigare okänt IgA enzym. I detta arbete beskriver vi det tidigare okända proteaset BspE (*Bdellovibrio* elastase-like serine protease) från den predatoriska bakterien *Bdellovibrio bacteriovorus*. Resultaten visar att BspE huvudsakligen klyver IgA på ett ställe och frisätter en liten del av proteinet som bär på en sockerkedja, vilket möjliggör specifik analys och av denna sockerstruktur. Dessa resultat bidrar till den basala kunskapen om *Bdellovibrio bacteriovorus* biologi och skulle möjligtvis kunna användas som ett forskningsverktyg för att titta närmare på biologiska aktiviteter av IgA.

I artikel III utreder vi det bakteriella enzymet EndoS, som klyver den konserverade sockerkedjan på IgG och därmed förlorar sin kapacitet att aktivera immunförsvaret, funktion under en infektion med *Streptococcus pyogenes*, och om denna aktiviteten bidrar till bakteriens förmåga att orsaka sjukdom. Genom att analysera patientmaterial från allvarliga *S. pyogenes* infektioner kunde vi visa att EndoS bidrar till bakteriens förmåga att orsaka sjukdom, men endast i närvaro av adaptivt immunförsvar som genererar specifika antikroppar mot bakterien.

I artikel IV undersöker vi om enzymatiskt inaktiva varianter av de bakteriella enzymen EndoS och EndoS2 kan användas för att specifikt binda och rena antikroppar ur komplexa biologiska material. Tidigare forskning har visat att EndoS och EndoS2 binder IgG specifikt och att denna interaktion i huvudsak är mellan enzymen och den konserverade sockerstrukturen på antikroppen. Genom att fästa inaktiva varianter av EndoS och EndoS2 på ett särskilt substrat kunde vi utföra olika experiment för att undersöka om, och under vilka förhållanden vi kunde rena upp antikroppar. Våra resultat visar att de inaktiva enzymen kan användas för att

specifikt rena upp humant IgG ur komplexa biologiska material som mänskligt blod, och att sockerkedjan på antikroppen är nödvändig för denna interaktion.

Denna doktorsavhandling belyser betydelsen av den konserverade kolhydraten på cirkulerande antikroppar (IgG), för ett välfungerande antikroppsmedierat immunsvar, samt hur den sjukdomsframkallande bakterien *Streptococcus pyogenes* undviker immunförsvaret genom att klyva sockerkedjan på IgG och därmed reducera antikroppens funktion. Arbetet som presenteras i denna avhandling belyser även hur EndoS och andra bakteriella enzymer kan användas som bioteknologiska verktyg för akademisk och industriell forskning, men även som verktyg för kvalitetskontroll av antikroppsbaserade läkemedel.

Acknowledgements

The years as a doctoral student has been a journey during which I have developed not only professionally but also personally. It is evident to me that the work presented in this thesis would not have been possible without the support and encouragement from all my supervisors, co-workers and friends, as well as my family.

To my supervisor

Mattias I hardly know where to begin. Thank you for your constant support and guidance throughout my studies, it has been invaluable! Your curiosity and passion for science has been contagious, and your unwavering confidence in me has always encouraged me to pursue my curiosity and that which I find interesting. The freedom and responsibilities I have been entrusted has allowed me to thrive and develop as a researcher. For this I will be forever grateful, and I will always look back at this time in my life with appreciation and joy.

To my co-supervisors

Rolf Starting with my Masters project at Rockefeller, you have been a constant and reliable, support throughout my studies. I am more than grateful for all our stimulating discussions and collaborations that continuously challenged and inspired me, and propelled me forward. I have always trusted your honest input and valued your opinions and advice. I also appreciate your patience when giving feedback on “final manuscript; draft 12.2”. It has been magnificent! For all of this I want to thank you.

Andreas I would like to thank you for all the support, enthusiasm, helpful input on experiments and writing, and all the interesting conversations about science and other stuff. Working with you has been exciting, challenging, stimulating and so much fun!

Oonagh I am truly happy that you took on the role as my co-supervisor, there were times where without you I would have been lost. I want to thank you for all your guidance and support! Working with you has been inspiring, and I have truly

appreciated all of our discussions and your scientific input and advice. Your door has always been open, and you have helped me to put things in perspective more than once.

I would also like to especially thank

Anita For everything you do, you are priceless! I have truly treasured our conversations, your positive energy, the constant support and care. For all of that and more, thank you!

Inga-Maria Thank you for your willingness and enthusiasm to discuss science and share your knowledge. I admire your spirit and passion for both science and travelling, I have truly appreciated our conversations on both these topics. There are now even more places in the world that I want to visit and explore.

Maria A Your unceasing support and cheerfulness has always brightened up the day. You have lifted my spirits more than once during times of failed experiments and frustration, and restored self-confidence and hope in that which feels impossible.

Jonathan S I would like to thank you for the time as my supervisor during a project leading up to the doctoral studies, maybe without even knowing it you taught me several important lessons that have stayed with me ever since. I have always appreciated your advice and encouragement.

To my co-authors and collaborators

Thank you for all your support and contributions, **Johan Malmström**, **Christofer Karlsson**, **Adam Linder** and **Henrik Molina**.

I want to thank everyone at the department for contributing to a positive atmosphere and enjoyable working environment. A special thanks to **Ariane**, **Frida**, **Wael**, **Martin**, **Therese**, **Sebastian**, **Marta, Gisela and Oscar** for all the support, advice and encouragement, not to forget all the laughs and discussions during coffee breaks, lunches and after-works. It has been so much fun and without all of you this journey would not have been the same. I am overjoyed and thankful to have made so many true friends that I hope will remain in my life always.

To my family and loved ones, thank you for your unfaltering support, encouragement, and unconditional love. You have always believed in me, and you made me believe in myself in times of doubt.

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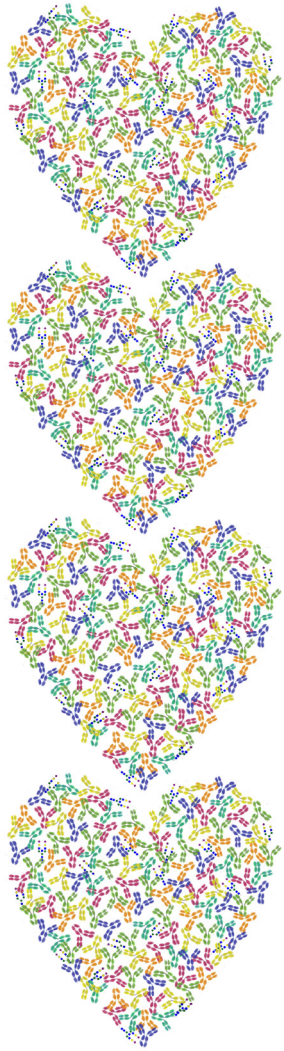
It is not in the stars to hold our destiny but
in ourselves

-William Shakespeare

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903



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FACULTY OF MEDICINE

Division of Infection Medicine
Department of Clinical sciences Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:100
ISBN 978-91-7619-829-2
ISSN 1652-8220