How antibody type and bacterial antigens influence complement activation

Writing Assignment – Literature Review

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1 Abstract

Antibodies play an important role in the anti-bacterial immune defence. One of these roles is the activation of the complement system, which is of major importance in antibacterial immunity. There are several aspects which determine how well an antibody can activate the complement system, which we discuss in this review. Here, the different antibody isotypes display distinct characteristics based on their size and structure, with IgG and IgM being the most potent complement activators. For instance, IgM is secreted as a pentamer or hexamer and has thus high avidity compared to IgG, which is secreted as a monomer. However, IgG can target more specific antigens and can reach more concealed antigens due to its smaller size. The size of the antibodies also influences their interactions with other proteins and antibodies, with larger antibodies like IgG3 being more accessible to other proteins like the complement protein C1q than the smaller IgG1, but at the same time also being less rigid. The differences between antibody isotypes and subclasses allows them to target different bacterial antigens. IgM with its high avidity and bigger size may be better suited to target larger, more exposed and more variable antigens like CPS and LPS, whereas IgG antibodies may be advantageous in targeting more specific, less protruding antigens like OMPs. Taken together, a complex interplay between antibody characteristics, determined by their size and structure, and bacterial antigen characteristics, like availability and density, influence how efficient an antibody can activate the complement system in response to bacterial infection.

Key words: Antibody isotypes, antibody structure, complement activation, bacterial antigens

2 Aim of the review

In this review, we aim to discuss how different antibody isotypes and subclasses differ in their ability to activate the complement system in response to bacterial antigens. Here, we will highlight recent advancements in the knowledge of antibody structure and how this relates to the binding of C1q and the activation of the complement cascade. This will be connected to different bacterial antigens, discussing how their characteristics influence an antibody's ability to activate complement. With this review, we want to provide an insightful overview over the topic, identifying gaps in knowledge and hopefully inspiring new research questions, with the aim of increasing knowledge and improving antibody therapy against bacterial infections.

3 Introduction

3.1 Antibodies

Antibodies, also known as immunoglobulins (Ig), are proteins that play an important role as part of the humoral immune response in battling pathogens and cancer. For instance, they neutralise and trap pathogens, and convey several different effector functions. Moreover, they form an important link between the adaptive and innate immune system (1,2). Among these effector functions are antibody-dependent cell-mediated cytotoxicity (ADCC) and opsonising targets to facilitate phagocytosis by phagocytic cells like macrophages (1,2). Another important effector function of antibodies, specifically those of the IgM and IgG isotypes, is their capacity to initiate the complement system through engagement and activation of the complement protein C1q. This induces a catalytic cascade resulting in inflammation, recruitment of immune cells, and killing of target cells, called complement-dependent cytotoxicity (CDC) (3,4).

There are five distinct antibody isotypes in humans, namely IgM, IgD, IgG, IgA, and IgE. All of these isotypes exhibit a Y-shaped form as monomers, consisting of two heavy chains and two light chains (5). They can be functionally divided into two antigen-binding fragments designated as the Fab domains, along with one crystallizable fragment known as the Fc domain. The Fab domains confer the specificity of the antibody towards its target antigen and thus its affinity. Affinity describes the binding strength between a Fab domain and its target, while avidity, also known as functional affinity, is the accumulated binding strength of the Fab domains of one antibody (6). Consequently, a higher number of Fab domains results in higher avidity. The Fc domain is unique for each antibody isotype and dictates their respective effector functions. Fab and Fc domains are connected by the flexible hinge region, which differs in size and flexibility between antibody isotypes (1).

IgM is the first antibody isotype to be expressed during infection (7,8). In its pentameric, secreted form, IgM is with 1000 kDa the largest antibody isotype (8). The pentameric structure is build up from five IgM monomers, of which two are linked via a joining chain (J chain) (8,9). The presence of the J chain allows for transportation across mucosal epithelia (5,7). Due to its multimeric nature, IgM shows high avidity, which effectively compensates for its rather low affinity. Thus, IgM is able to opsonise its target antigen, thereby marking it for other immune cells, and to induce complement activation (1,5,7). IgG is the most prevalent antibody isotype and has a molecular weight of on average 150 kDa (1,5). It can be further divided into the four subclasses IgG1 to IgG4 (1), all of which show different capacities to activate the complement system (5,10). Due to its long half-life and increased antigen affinity and specificity, IgG is critical in pathogen clearance. Specifically, the ability of IgG to induce effector functions through IgG-Fc tail receptors (FcγRs) located on various immune cells like Neutrophils

and NK-cells are of importance (11). Notably, IgG also possesses the unique ability to cross the placenta, endowing it with a crucial role in the protection of neonates and babies.

3.2 Bacterial antigens

Bacteria can be classified into Gram-positive and Gram-negative species, which differ greatly in the composition of their cell wall, resulting in distinct characteristics. In both Gram-negative and Gram-positive bacteria, the protein peptidoglycan (PGN) forms the backbone of their cell wall. PGN is made up from repeating linear units of disaccharide N-acetylglucosamine linked to N-acetylmuramic acid (12). However, the Gram-positive and the Gram-negative bacterial cell wall differ greatly in the thickness of their PGN layer (12,13). In Gram-positive bacteria, the PGN layer is on top of the cytoplasmic membrane and can reach a thickness of 30 to even 100 nanometres, providing mechanical stability (12,14). Another important structure in the Gram-positive bacterial cell wall is teichoic acid (TA), which reach through and stick out of the PGN layer. There are lipid TAs (LTAs), which are anchored in the cytoplasmic membrane via lipid tails, and wall TAs (WTAs), which are covalently bound to the PGN molecules (12). TAs are surface glycopolymers that are highly negatively charged due to phosphate groups, and thus form a repellent against antimicrobial peptides. Interestingly, while LTA structures are relatively conserved across different Gram-positive bacterial species and strains, WTAs are very variable in their structure (12).
Gram-negative bacteria have a differentially build cell wall. On top of the cytoplasmic membrane, there is only a thin PGN layer, with a second membrane on top, the outer membrane (OM). The space in between the two membranes is called

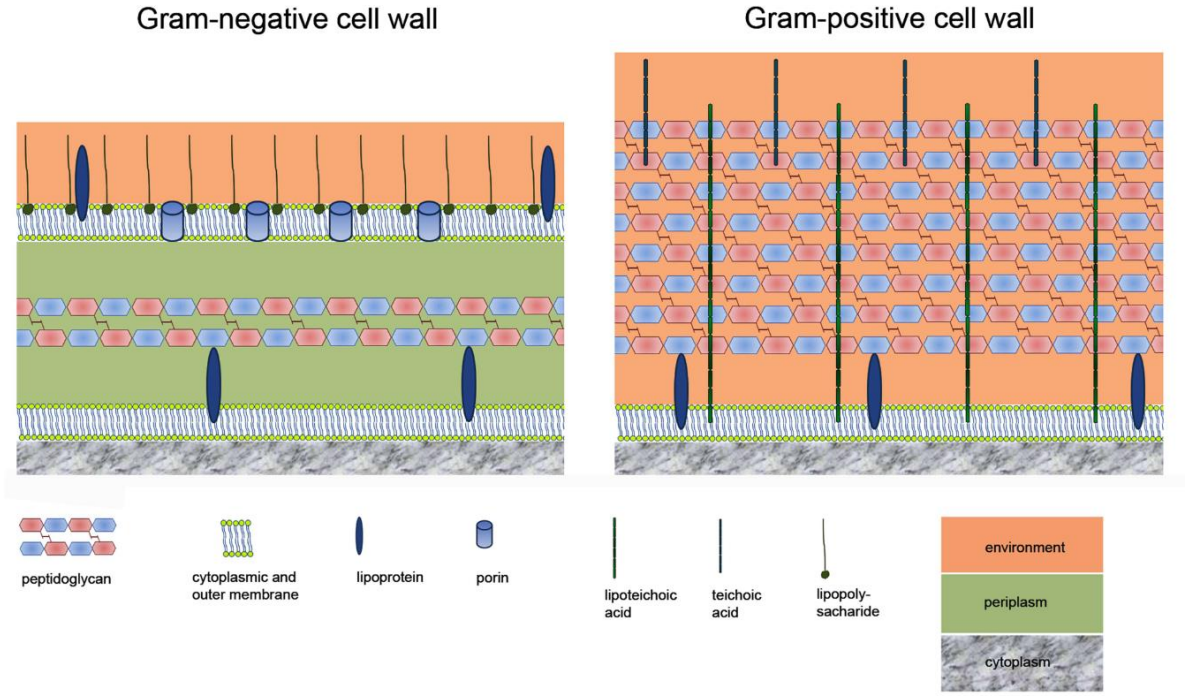

Figure 1: Schematic representation of the Gram-negative and the Gram-positive bacterial cell wall.

The Gram-negative bacterial cell wall is characterised by the presence of two membranes, the inner, cytoplasmic membrane, and the outer membrane. In between the two membranes is the periplasmic space, in which a thin layer of peptidoglycan can be found. There are lipoproteins present in the cytoplasmic and the outer membrane, while lipopolysaccharides and porin proteins are only found in the outer membrane. The Gram-positive cell wall consists of only one membrane, the cytoplasmic membrane, and a peptidoglycan layer that is much thicker than the Gram-negative peptidoglycan layer. There are lipoproteins and lipoteichoic acid present in the cytoplasmic membrane and teichoic acid, referred to as wall teichoic acid in the main text, linked to the peptidoglycan layer. Both lipoteichoic acid and teichoic acid protrude from the peptidoglycan layer. Figure taken from Rohde et el., 2018 (12), copyright licence ID: 1438520-1.

periplasm or periplasmic space (12,15,16). This composure allows for communication with the external environment through selective pores and transporters within the OM, while simultaneously acting as a physical barrier against antimicrobial peptides (12,15,17). These pores and transporters are part of a group of proteins called outer membrane proteins (OMPs), which often serve bacterial immune evasion (17,18). Another noteworthy component of the outer membrane are lipopolysaccharides (LPS), which hold substantial immunological value (15,19,20). LPS is made up of a hydrophobic membrane anchor portion, lipid A, and a non-repeating core oligosaccharide coupled to a distal polysaccharide, the O-antigen (O-ag) (19,20). While LPS can protect the bacteria from detection by the immune system, bacteria can also be directly targeted and detected by LPS. For instance, lipid A can be recognised by the Toll-like receptor 4 (TLR4), leading to the induction of inflammation via NFκB (19,20). Lipid A as well as O-ag are highly variable across different Gram-negative bacteria, which greatly alters their pathogenicity and susceptibility to immune effector functions (15,20).

In addition to the bacterial cell wall, Gram-positive and Gram-negative bacteria can have a polysaccharide capsule surrounding them (21,22). This capsule is typically composed of only one polysaccharide, the capsular polysaccharide (CPS) (21). It can form a tight barrier, and thus protect the bacteria from for instance antimicrobial peptides or complement proteins, making it a highly adaptable and thus variable protector of the bacteria from external factors (21,23,24).

3.3 Complement activation

The complement system is a complex network comprising roughly 50 serum proteins and cell surface receptors. When activated, the complement system triggers a series of immune responses through a proteolytic cascade (25,26). This cascade is initiated via the recognition of pathogenic surfaces, either through pattern recognition receptors (PRRs) that are part of the complement system, or through antibodies (26,27). The primary effector functions of the complement system are opsonising pathogens or aberrant cells and facilitating their clearance by phagocytic cells like macrophages. Moreover, the complement system recruits immune cells to sites of infection and induces inflammation via chemoattractants and anaphylatoxins. One direct effector function is the elimination of for instance Gram-negative bacteria through the formation of membrane attack complexes (MAC), which are membrane-penetrating pores (26–28).

The complement system can be activated through three distinct pathways - the classical pathway, the alternative pathway, and the lectin pathway. All three pathways exhibit partial overlap and result in the formation of C3 and C5 convertases and engage in positive feedback loops (25,26). The lectin pathway (LP) is activated when its intrinsic PRRs like mannose-binding lectin (MBL) and ficolins recognise a foreign surface antigen. Then, a proteolytic cascade is triggered, in which the MBL-associated serine proteases (MASPs) cleave C2 and C4 and thus form the C4bC2b C3 convertase (27). The alternative pathway (AP) can on the one hand serve as an amplification loop, since factor B and factor D can interact with membrane bound C3b, and consequently form the C3bBb C3 convertase (25–28). On the other hand, the AP can serve as a third initiating pathway, seeing as spontaneous hydrolysation of C3 into hydrolysed C3 ($C3_{H2O}$) is attributed to the AP. $C3_{H2O}$ is structurally similar to C3b and can form a C3 convertase with factor B and D (26,27). However, the exact mechanism of this remains unknown.

The classical pathway (CP) is initiated when the complement protein C1q binds to the Fc region of complement-activating antibodies, such as IgM and IgG, which are attached to the surface of pathogens or aberrant cells. This binding triggers a series of events involving serine proteases C1r and C1s, which are complexed with C1q and become autocatalytically activated. These proteases, in

turn, cleave C4 and C2, producing large fragments C4b and C2b and small fragments C4a and C2a. The larger fragments combine to form the C4bC2b C3 convertases like in the LP, which can cleave C3 into its larger fragment C3b and the smaller fragment C3a. C3b attaches to the pathogenic or aberrant surface, opsonising it, and, if present in sufficient density, prompts the C3 convertases to shift their substrate to C5. This leads to the deposition of C5b on the pathogenic or aberrant surface and the initiation of the formation of the C5b-9 MAC complex. One C5b-9 MAC complex consists of C5b, C6, C7, C8 and up to 18 C9 molecules (27). Simultaneously, the smaller fragments C3a and C5a act as potent chemoattractants and anaphylatoxins, inducing inflammation and recruiting immune cells (26–28).

4 Hexameric and pentameric IgM in complement activation

4.1 Secretory IgM can form pentamers or hexamers

Secretory IgM is generally thought to have a pentameric structure supplemented by a J chain (29). However, alternative forms of secretory IgM, lacking a J chain, have been described. These alternative forms of secretory IgM predominantly assemble into hexamers, although smaller polymers have also been suggested (29) (Figure 2). Hexameric IgM (IgMh) was initially discovered in diseases such as Waldenström macroglobulinemia or cold agglutinin disease, but the natural prevalence of IgMh is still highly debated (30). Some studies estimate that IgMh accounts for approximately five percent of total secretory IgM (31,32), while a more recent study suggests that natural secretory IgM almost primarily exists in its pentameric form, with IgMh only becoming apparent under pathological conditions (8). This is an important aspect to be kept in mind when studying secretory IgM *in vitro* and wanting to mimic natural conditions. In this case, the J chain needs to be co-expressed with IgM to achieve the formation of pentameric IgM (IgMp) (29).

4.2 Complement activation by pentameric and hexameric IgM

Regardless of their prevalence, both IgMp and IgMh are known to be potent activators of the complement system (33,34). This activation is achieved through binding of the complement protein C1q to the Fc tail, which activates the C1-complex. Notably, C1q possesses six Fc binding domains, and multimerisation of antibodies has been shown to be essential for C1 activation (29). This highlights why IgM is such a potent activator of complement, seeing as it naturally provides such structural obligations. In contrast, IgG is secreted as a monomer and must first undergo oligomerisation to effectively bind C1q (29,33,35,36).

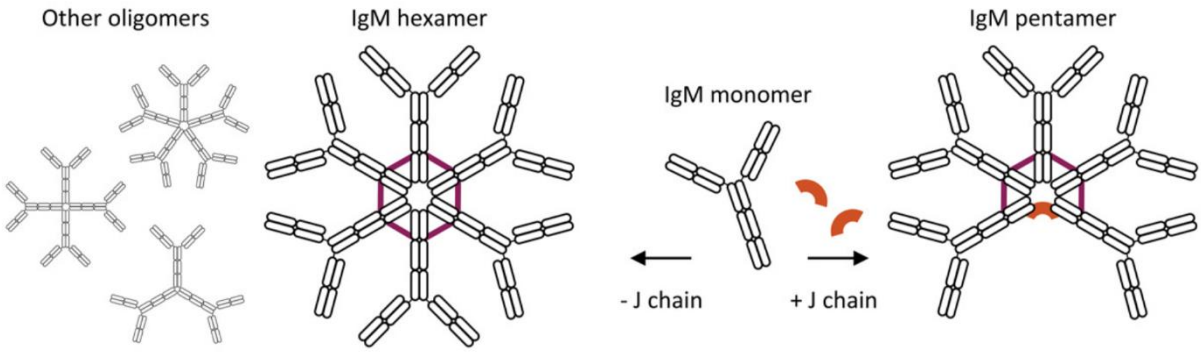

Figure 2: Schematic representation of secretory IgM, which forms pentamers in the presence of the J chain (orange), and hexamers or other, smaller oligomers in the absence of the J chain.

In its pentameric form, the J chain takes the position of the sixth IgM monomer, resulting in a hexagonal arrangement. Figure adapted from Oskam et al., 2022 (29).

A crucial aspect of complement activation by IgMp and IgMh is that the C1q binding sites on the Fc domain only become accessible upon recognition and binding of the respective antigens by the antibodies. This mechanism is speculated to prevent spontaneous complement activation (7,33), and can be attributed to structural components of polymeric IgM. Both IgMp and IgMh form an asymmetrical pentameric planar structure. Herein, the J chain replaces the sixth IgM monomer in IgMp (8,9). Although both arrangements are planar when in solution, they transform into hexagonal, staple-shaped structures upon binding to their respective surface antigens. The conformational change allows other Fab arms of the polymer to bind in a pair wise manner, contributing to the high avidity of IgM polymers (34). A recently discovered hinge region in the Fc part of IgM allows the Fab domains to pivot both in and out of plane. This enables them to move together, not singularly, to find and bind their antigen (31). Furthermore, the conformational change results in the formation of an Fc-platform, exposing the amino acid sequence DLPSP on the Fc domains of the IgM polymers. This sequence can now be bound by globular C1q (gC1q), the Fc binding domain of C1q (34). This shows that conformational changes within the IgM polymer upon antigen binding allow for complement activation through interaction with C1q.

4.3 Potency of IgMp and IgMh in complement activation

Recent studies have shown that IgMh, especially under conditions of low antigen availability and density, shows significantly greater potency in complement activation compared to IgMp (29). This may be due to several factors. Firstly, IgMh possesses a higher avidity than IgMp due to its 12 Fab domains, as opposed to the 10 Fab domains in IgMp. This may result stronger binding or better compensation for variable antigen targets. Secondly, C1q itself is a hexameric protein. Consequently, when C1q binds to IgMh, it achieves a stronger binding than with IgMp, where only five out of the six Fc binding domains of C1q are engaged (34). Consequently, complement activation by IgMh benefits from IgMh's high avidity and superior binding strength of C1q to IgMh.

The enhanced reactivity of IgMh could be beneficial in clearing difficult infections. On the other hand, IgMh has been shown to display higher haemolytic activity than IgMp and is supposedly connected to autoimmune diseases like cold agglutinin disease (9,32). Nevertheless, the role of IgM autoantibodies in the development of autoimmune diseases is generally not clear (37). Moreover, other antibody isotypes such as IgG are also involved in autoimmune diseases like systemic lupus erythematosus (38). Therefore, it is essential to emphasise that any statements regarding the potential harm of IgMh are mere speculation at this point. To clarify the safety of IgMh and IgM in general within the human body, further investigations regarding their potential to induce autoimmune reactions should be conducted. As the safety of the patient is of paramount importance during antibody therapy, future research for the usage of IgM in therapeutics should focus on IgMp. Its natural occurrence is a strong argument for its safety within the patient, while providing much of the same advantages as IgMh.

5 IgG subclasses in complement activation

5.1 IgG can be divided into four subclasses

The antibody isotype IgG is made up of four subclasses: IgG1, IgG2, IgG3 and IgG4 (Figure 3). These subclasses primarily differ in the Fc region, particularly the upper CH2 domains, and the hinge region of the antibodies (11). While the Fc region in general confers interactions of the antibody with other proteins or receptors, the hinge region is a flexible linker between the Fab and Fc region of antibodies (11). This means that it gives the an-

tibody important steric characteristics regarding flexibility or rigidity, greatly influencing the interactions of the Fc tail. Seeing as the hinge region and the Fc region are what sets the different subclasses apart, the IgG subclasses also significantly differ in their effector functions.

IgG1 constitutes approximately 60 % of all IgG antibodies (11), making it the most abundant subclass. Its hinge region is with 15 amino acids (AA) of medium flexibility and C1q shows the second strongest binding to the IgG1 Fc tail out of all IgG subclasses. In general, IgG1 shows relatively strong binding to nearly all IgG Fc-gamma-Receptors (FcγR) (11), which stresses its importance in several immune responses, for example against soluble or membrane proteins (39). Furthermore, IgG1 plays an important role in immune protection of infants through placental transfer (11). The IgG2 subclass is most abundantly produced in the antibody response towards polysaccharides (39). This makes it essential for immunity against capsulated bacteria like *Streptococcus pneumoniae*, where it is the only antibody isotype able to effectively opsonise those bacteria (40). This also becomes apparent in the high number of recurring infections with encapsulated bacteria in children under the age of two, where IgG2 production is low (40). However, IgG2 binds poorly to FcγRs and C1q, rendering it relatively weak in the induction of effector functions like complement activation or ADCC (41). IgG3 is the largest of the four subclasses with a molecular weight of 170 kDa. Its size is due to an exceptionally long hinge region made up of 62 AA (11), which renders IgG3 very flexible. Moreover, IgG3 is bound strongest by C1q and by several FcγRs out of all IgG subclasses and is overall very effective in the induction of effector functions, including ADCC. It makes up for only about four percent of all IgG antibodies and has a relatively short half-life (11). This may be to limit its potent pro-inflammatory characteristics, which is however only speculative. Together with IgG1, IgG3 is often induced by viral infections (39). Lastly, IgG4 only makes up for a small portion of IgG antibodies with roughly four percent (11). Its production is typically induced by allergens, prolonged exposure to antigens without the induction of disease, or by parasites (11,42). Its affinity towards FcγRs is of medium strength, while C1qs affinity for IgG4 is particularly weak (11). Since this review is focused on the complement system, we will in the following parts focus on the capacity of the IgG subclasses to activate the complement system and leave FcγR-induced effector functions out of the picture.

5.2 Oligomerisation of surface-bound IgG induces complement activation

IgG in general has been shown to be able to activate complement through the classical pathway, but there are differences between the distinct IgG

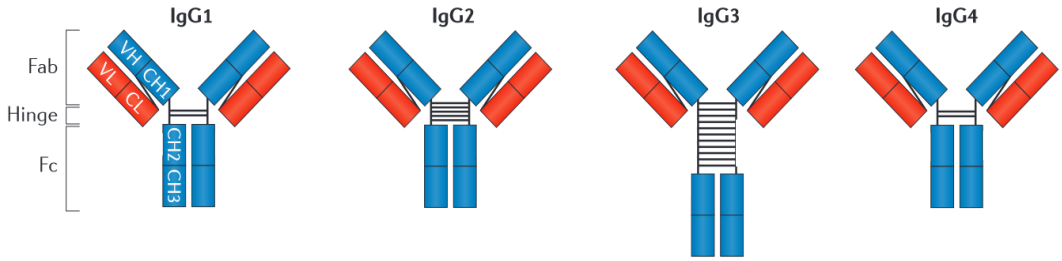

Figure 3: Schematic representation of the structural composition of the different IgG subclasses IgG1, IgG2, IgG3 and IgG4.

The light chain is depicted in red and the heavy chain in blue, which can each be further divided into the variable (VL and VH) and the constant domains (CL and CH). The Fab (antigen-binding fragment) region and the Fc (crystallisable fragment) domain are linked via the flexible hinge region, which consists of disulfide bridges (-). The subclasses mainly vary in their constant domains and in the length of their hinge region, with IgG3 having the longest, and IgG1 and IgG4 having the shortest hinge region. Figure adapted from Lu et al., 2017 (1).

subclasses (25–28). Generally, IgG1 and IgG3 are the strongest activators of complement amongst the IgG subclasses (10,43). This is reflected in the binding strength of C1q towards them (11). Fittingly, C1q shows much weaker binding affinity towards IgG2 and IgG4. This seems to be largely due to point mutations in the amino acids responsible for C1q binding within the CH2 region of the antibodies (44,45). This does however not mean, that they have no capacity to activate complement at all. For a long time, IgG4 was believed to not be bound by C1q at all and therefore not activate the C1-complex (11). Yet, a recent study has shown that IgG4 is able to activate complement, but only in the context of high density of antigens and a high concentration of antibodies (10). Since this is a rather new discovery, it still needs further investigation. While IgG2 seems to not play an important role in complement activation through protein antigens, it is of importance in complement activation through high density epitope polysaccharides (46). Nevertheless, this review will in the following focus on the more potent complement activators IgG1 and IgG3 and will not address IgG2 and IgG4.

5.2.1 Antigen binding influences inter-antibody interactions

In general, antibodies can bind their respective antigen in a monovalent or bivalent manner. When only one of the two Fab arms of an antibody is engaged in antigen binding, while the other Fab arm is unattached, the antibody is bound monovalently. If both Fab arms of an antibody are engaged in binding, it is bound bivalently. Whether an antibody is bound mono- or bivalently has great influence on its capacity to turn, move, and interact with other antibodies. When attached with only one arm, the rest of the antibody can move much more freely than when attached with two arms. Since interaction of antibodies with one another and with receptors is an important aspect of their immune function, the ability to bind mono- or bivalently is of great importance. For instance, activation of the complement system by antibodies requires them to cluster together via their Fc domains through a process called oligomerisation (36,47).

5.2.2 Oligomerisation of IgG antibodies

Since oligomerisation is necessary for complement activation, the oligomerisation properties of IgG have been investigated thoroughly (10,35,36,47). It has been found that oligomerisation of IgG occurs through specific noncovalent interactions of the Fc domains of the partaking IgG monomers (36,47). Seeing as the affinity of the Fc domains towards one another is relatively low, spontaneous oligomerisation in solution is very rare. Instead, oligomerisation of IgG is mainly dependent on clustering of the antibodies on the cell surface they are bound to. There are two theories as to how this comes to happen, which have been experimentally investigated (47). One is called the horizontal pathway, which describes the oligomerisation of two or more antibodies that are bound to the same surface. These antibodies collide and bind spontaneously via diffusion within the surface they are bound to. The other pathway is known as the vertical pathway (47). It implies that surface-antigen bound antibodies recruit unbound, soluble antibodies through their Fc tails. The recruited antibodies will then be in close proximity to the surface, rendering them more likely to bind their surface-antigen as well.

While both IgG1 and IgG3 are able to bind to their antigens in a mono- and bivalent manner, their capacities to interact with other antibodies and therefore oligomerise differ from one another (47). When only one of their Fab arms is engaged in antigen binding, they can both interact with other antibodies, therefore oligomerise following the horizontal and the vertical pathway, and subsequently activate complement. When both of their Fab arms are antigen-bound, this paints a different picture. IgG3 is still able to interact with other antibodies through its Fc tail as described in

the horizontal and vertical pathway. This is due to the long and flexible hinge region of IgG3, which allows for such interactions (11,35). IgG1, however, has a smaller, less flexible hinge region (11). Consequently, in bivalently bound IgG1, the Fc tail of the antibody can no longer interact with other antibodies as described in the horizontal pathway. It may yet still recruit new antibodies as seen in the vertical pathway. However, to truly engage into oligomerisation, IgG1 needs to interact with neighbouring, antigen-bound antibodies and thus most likely needs to loosen one Fab binding. This means that IgG1 can mainly engage in the formation of oligomers, and thus complement activation, when monovalently bound. This has consequences for the avidity of IgG1 and IgG3 oligomers. Since IgG3 monomers can be bound bivalently while forming an oligomer, more Fab arms can be engaged in binding (35). Additionally, bivalently bound antibodies are able to form highly organised Fab clusters on the membrane they are bound to, which monovalently bound antibodies cannot. This increased antibody density enables them to form more oligomers on the same space in comparison to monovalent oligomers (35,47). Concluding, oligomers formed by bivalently bound antibodies like IgG3 cannot only form at higher densities, but also show stronger avidity than their facultative monovalent equivalents formed by IgG1.

5.2.3 The role of oligomerisation in IgG dependent complement activation

It has been mentioned several times that oligomerisation of IgG is essential for complement activation by IgG. The reason for that is that the first step in complement activation through antibodies is the binding of C1q (27,28). While the C1q binding domain on the Fc tail of IgG monomers is exposed at all times, in contrast to IgM, spontaneous binding of C1q to IgG monomers is very rare (36,47). This is due to the low affinity of C1q to a single C1q binding domain on the Fc tail of an IgG. This must be compensated by the accumulation of avidity. Which is achieved through oligomerisation of IgG. It has previously been speculated that smaller IgG oligomers containing three or four monomers were sufficient to activate complement (36). However, one study, focused on the structure of IgG and its interaction with C1q, has shown that C1q binding and activation is only successful for IgG pentamers or hexamers, not smaller oligomers (47). This is due to two reasons. Firstly, IgG oligomers of four or less monomers do not accumulate sufficient binding strength of C1q towards them to properly engage C1q. Secondly, binding of C1q to Fc domains induces a conformational change within C1q that allows C1s and C1r to initiate the complement cascade (47). This conformational change is only achieved when at least five out of the six gC1q Fc binding domains are engaged. However, another study stated that as little as two IgG monomers are enough to activate the C1-complex, even though complement activation was more efficient with higher order oligomers (48). When closely examining the two studies, one can speculate that these differences may arise from differential experimental set ups, with one study having much more controlled C1q binding conditions (48) than the other (47). Summarising, it still needs to be clarified whether oligomerisation of IgG to pentamers or hexamers is necessary for complement activation via the classical pathway compared to smaller oligomers, not only to achieve proper binding of C1q, but also the conformational changes within C1q that induce the complement cascade.

5.2.4 Implication for antigen binding, oligomerisation and complement activation in antibody therapeutics

Taken together, oligomerisation of IgG is vital to IgG induced complement activation. Seeing as bivalently bound antibodies can form oligomers at greater density than monovalent oligomers, this would lead to the assumption that bivalent oligo-

mers formed by IgG3 are more potent at activating complement than monovalent IgG1 oligomers. However, the matter seems to be more complicated than that. For IgG3 and IgG4, bivalent oligomers have been shown to be more potent complement activators (10,35). On the other hand, enforcing monovalent binding for IgG1 through creating bispecific IgG1, IgG1 with two Fab domains specific for different antigens, increased complement activation (36). Thus, it seems that whether the formation of bivalent oligomers at high density or the formation of monovalent oligomers at lower density is advantageous for complement activation depends entirely on the IgG subclass. In respect to designing future antibody therapies, the advantages and disadvantages of mono- or bivalent binding of different IgG isotypes and possibly chimera in respect to oligomerisation and complement activation should be studied more extensively. Since, however, there are more factors coming into play regarding complement activation than IgG oligomerisation, these factors shall be discussed hereafter.

6 Proximity to the membrane – why distance matters

6.1 The Fc platform and its implications for C1q binding

Since it has been shown that antibody oligomerisation is essential for C1q binding and thus complement activation, the Fc platform formed by these oligomerised antibodies plays an important role (29). This platform is made up of the assembled Fc domains of the antibodies forming an oligomer and provides the binding or docking station for C1q. There are multiple aspects which can influence the binding of C1q to this docking station, notably the stability and the accessibility of the Fc platform. These two factors are often closely connected. For instance, IgG3 Fc platforms exhibit reduced rigidity due to the elongated and flexible hinge region of IgG3, resulting in decreased stability (35,49). However, this also positions the IgG3 Fc-platform around 22 nm away from the membrane that it is bound to, representing the most extended Fc-platform out of all antibody isotypes and subclasses (35). This results in great accessibility of the IgG3 Fc platform for C1q, seeing as the platform might protrude out of the corona of proteins covering the surface the antibodies are bound to (35,49,50). Moreover, this might explain in part why several studies have shown that C1q has the strongest affinity for IgG3 amongst all IgG isotypes (32,51,52). The Fc platform of IgG1 oligomers is located much closer to the surface of the membrane they are bound to with around 11 nm due to the reduced length of the hinge region of IgG1 (11,35). This may obscure the Fc platform to C1q binding by surrounding proteins, does however lead to greater stability of the platform. Even though IgM has a Fc domain made up of three rather than two subunits (53), antigen-bound IgM oligomers adopt a staple-like conformation, reducing the height of the Fc platform to approximately 13.5 nm above the membrane (8,9,33,35). This might also lead to the obscuration of the IgM Fc platforms by surface proteins like IgG1 Fc platforms. Additionally, the recent identification of a hinge-like region in IgM monomers, which is less flexible than a normal hinge region, confers increased rigidity to IgM Fc platforms (31). Overall, IgG3 has the highest, but also least stable Fc platform due to its long hinge region, while IgG1 and IgM Fc platforms are located much closer to the membrane the antibodies are bound to. This gives them greater stability but might also obscure the Fc platforms from C1q binding.

Having established the considerable variation in stability and accessibility of Fc platforms across different antibody isotypes and subclasses, this paragraph will discuss how this impacts C1q binding and thus complement activation. There are conflicting reports about how flexibility and rigidity influence C1q binding. Some studies report that more flexible antibodies with a longer hinge region like IgG3 show increased C1q binding (50). They argue that, in more rigid antibodies like

IgG1 or IgM, unbound Fab arms might block the C1q binding site on the Fc domain of the antibodies and thus prevent C1q binding. However, other studies state that a more rigid hinge region favours C1q binding (54–56), possibly due to stronger binding interactions. In light of these contradictory findings, further investigations to clarify the complex interplay between rigidity and flexibility of antibodies regarding C1q binding is essential. In the end, it may very well be a balancing act that needs better understanding in order to harness its full potential regarding the design of therapeutic antibodies.

6.2 Proximity to the membrane and C4b deposition

The distance of the Fc platform of antibody oligomers not only influences their accessibility for and binding of C1q, but also the deposition of C4b. This is a key aspect in the initiation of the complement cascade, seeing as C4b is part of the C3 convertase of the classical pathway (26,27,32). The deposition of C4b by C1-complexes bound to IgG1 oligomers or IgM occurs directly onto the surface of the membrane (34,35). This is thought to be the usual placement for C4b, where it forms the C4bC2b C3 convertase (26,27). While the smaller size of IgG1 allows for the direct deposition of C4b onto the membrane, the deposition mechanism for IgM involves different factors. Specifically, the C1q collagen helices attached to an IgM Fc platform are more widely spread compared to those bound to an IgG Fc platform, due to the larger surface area of the IgM Fc platform (34). This spacing brings the serine protease C1s and C1r of the C1-complex closer to the membrane, allowing for direct deposition of C4b onto the membrane. However, this is not the case for IgG3. Recent findings indicate that an activated C1-complex bound to an IgG3 Fc platform deposits C4b not on the membrane but on the IgG3 molecule itself (35). It remains yet unclear as to how this affects the further progress and regulation of the complement cascade. On the one hand, soluble complement regulators could have better access to C4b deposited on IgG3 instead of the membrane. On the other hand, surface bound complement regulators might not reach C4b any longer. Additionally, this mode of deposition might interfere with the formation of the C3 convertase and the progression of the complement cascade on the target membrane. These differences might explain why IgG1 has been shown to induce stronger CDC than IgG3 (32,51). In IgG1 and IgM, complement initiation involves a single-step C4b deposition onto the membrane, whereas in IgG3, it probably involves an additional transfer of C4b from the antibody to the membrane. How this happens, how long it takes, and what it implies for the progress of the complement cascade remains yet to be elucidated. Ultimately, the distance of the Fc platform from the membrane is indeed a critical factor in C4b deposition. Future research should focus on understanding the implications of C4b deposition on IgG3 itself, particularly concerning the regulation and modulation of the complement cascade.

7 The influence of antigens in antibody-dependent complement activation

7.1 The perfect antigen – how antigens influence antibody effector function

The efficacy of antibody-mediated complement activation against bacteria is influenced by several factors related to the respective antigen or epitope (32). Key factors include the antigen's size, density, availability, orientation, geometry, and proximity to the bacterial surface. Bigger antigens, while more accessible, may influence optimal C4b deposition for complement cascade initiation due to the increased distance to the membrane, as is the case for increased distance to the membrane due to antibody size (Chapter 6.2). This is supported by findings that antigens proximal to the membrane enhance complement activation in

comparison to distal antigens (29,57). Another aspect is antigen density, which is crucial for antibody dependent complement activation, where again differences between antibody isotypes and subclasses have been observed (11,29,35). Thus, it seems like there is a delicate balance in optimal antigen distance to the membrane and antigen density, potentially influenced by antibody isotype, which needs to be further investigated. Moreover, the antigen's conformation, which affects its orientation and geometry, also has great influence on antibody interactions. The orientation of the antigen will influence the orientation of the antibody when binding it, including the Fc tail, which may either favour or hinder further interactions with other proteins or receptors (58,59). Furthermore, a recent study has demonstrated the presence of different patches on *E. coli*, some rich in OMPs and some rich in LPS (60). These differentially composed patches may result in different susceptibility to complement effector functions depending on the location on the surface of the bacteria. This hypothesis is supported by findings linking complement activation of anti-CD20 monoclonal antibodies (mAbs) in lymphoma treatment to segregation of the membrane into lipid rafts (61). However, this still must be proven to be translatable to bacteria. Altogether, multiple factors must be considered when evaluating antibody targets for complement immune response effectiveness, among which are antigen density, distance to the membrane and orientation.

7.2 Bacterial antigens in antibody induced complement activation

Antibody-dependent complement killing is a vital part of the innate immune response against bacterial infection and also of great interest in the development of therapy against bacterial infection (27). Thus, understanding the interactions between bacterial antigens, antibodies and the complement system is crucial. Here, especially the bacterial cell wall antigens of Gram-negative bacteria, like LPS or O-ag, and Gram-positive bacteria, like PGN or TA, are of special importance due to their high exposure (12,14,19,20). The differences between the build of the cell wall between Gram-negative and Gram-positive bacteria influences their susceptibility to certain effector functions. For instance, Gram-positive bacteria are thought to be resistant to MAC-induced killing (62). However, the general principles described in Chapter 7.1 apply to both.

While capsules are typically found in Gram-negative bacteria, they are also present in some Gram-positive bacteria like group B Streptococci (GBS) and *Streptococcus pneumoniae*. These capsules, being the outermost layer of the bacteria, are important targets for the immune response, and capsular polysaccharides (CPS) are among the most effective targets for vaccinations (63). However, the high exposure of CPS to the immune system leads to great selective pressure on CPS. Consequently, there is a very high variability among CPS of different bacterial strains (64). In some Gram-negative bacteria like *E. coli*, CPS and the K-antigen, a polysaccharide side chain of CPS, are thought to provide resistance against antibody binding and complement initiation by sterically hindering the access of antibodies and complement proteins to the bacterial membrane (65–70). Conversely, anti-CPS antibodies have been shown to mediate protective immunity against GBS (71). This is probably due to differences in the antigenic sites targeted by anti-CPS antibodies in different bacterial strains, which has great influence on the factors discussed in chapter 7.1. Given the conflicting effects of CPS as an immune target for antibodies and the complement system, further research is needed to assess the effectiveness of targeting CPS antigens for complement-driven immune responses.

LPS is, similar to CPS, a highly exposed and thus highly variable antigen (15,20). In *Klebsiella pneumoniae*, LPS and O-antigen (O-ag) reportedly inhibit antibody-dependent complement activation, likely by blocking access to antigens and the cell membrane (72,73). However, antibodies

targeting O-ag are known to confer protection against *Vibrio cholerae* and can facilitate antibody-dependent complement killing (74,75). Contrastingly, in *Neisseria meningitidis* the presence or absence of LPS appears to be irrelevant to complement activation, as both wild type and LPS knockout strains activate complement to the same extend (76). Yet again, differential antigenic sites targeted by antibodies on LPS in different bacterial strains might be the reason for this discrepancy in susceptibility to the complement system. Moreover, in some bacteria like *Neisseria meningitidis*, targeting OMPs rather than LPS seem to result in higher complement activation (77). Consequently, optimal antigen targets for complement activation vary greatly between different species of Gram-negative bacteria and need to be investigated more closely.

In Gram-positive bacteria, peptidoglycan (PGN) is the predominant antigen. It is conserved across various species, making it a prime target for immune response (11). However, so far, only PGN from *Staphylococcus aureus* has been shown to activate the classical complement pathway, while PGN from other bacterial strains like *Streptococcus pneumoniae* does not (78–82). Thus, it may be that PGN from different bacterial species poses different antigen targets for antibodies much like for LPS or CPS do, resulting in different immune responses. Antibodies targeting other Gram-positive bacterial cell wall components like protein A and TA have also been shown to activate the classical complement pathway in *S. aureus* (81), with TA being the most potent activator among them (82). While it might be that *S. aureus* is simply one of the more potent CP complement activators among Gram-positive bacteria, there is also a lack of research on this topic in other Gram-positive bacterial strains. The lack of knowledge about antibody-dependent complement activation in Gram-positive bacteria other than *S. aureus* might be partly due to Gram-positive bacteria being resistant to MAC-induced killing (62). Nonetheless, the pro-inflammatory and opsonizing capabilities of the complement system play a crucial role in the immune response against Gram-positive bacteria and should not be underestimated.

In conclusion, a deeper understanding of bacterial antigens in relation to complement activation is crucial to effectively harness the potential of the complement system in fighting bacterial infections. The significance of the complement system in immune responses against bacteria is highlighted by the very existence of bacterial complement resistance mechanisms. One example is the staphylococcal protein A in *S. aureus*, which binds to the Fc domain of certain IgG subclasses, inhibiting their oligomerization and thus complement activation (83). Such evolutionary mechanisms hindering complement activation, even if indirectly, demonstrate the critical role and effectiveness of the complement system in anti-bacterial immunity. While most current mAbs in bacterial therapy are targeted against exotoxins (63), recent advancements in the understanding of complement activation, antibody characteristics, and antibody engineering have opened new opportunities for targeting bacterial antigens, promising more effective strategies in bacterial infection management (8,29,31,34,35,47,84).

8 Affinity versus Avidity in immunity and therapy

In this review, different antibody isotypes in complement activation were discussed, emphasising the role of structural differences, antigen binding, and interaction with C1q. Here, affinity, the binding strength of a single Fab domain towards its antigen, and avidity, the accumulated affinity of all Fab domains within an antibody, will be considered. Both have great impact on the characteristics of an antibody and determine their binding capacities and thus the antigens they are able to bind to (6).

Pentameric or hexameric IgM demonstrate distinct advantages in avidity due to a greater number of Fab domains compared to monomeric IgG (7,33). However, most IgM naturally has a lower

affinity towards its antigen, seeing as it has not undergone affinity maturation (7,85). On the one hand, this may be a disadvantage for binding very specific antigens. On the other hand, it enables IgM to bind more variable and generic antigens like CPS or LPS. This fits with the natural appearance of IgM as the first antibody isotype of the antibody immune response, somewhat bridging between the general PRRs of the innate immune system and the more targeted adaptive immune system (7,8). Here, the high avidity of IgM can compensate for low affinity binding due to variations in the antigen (7). This might explain why most mAbs in development against CPS are IgM-based, and why anti-O-ag IgM is more effective at inducing CDC than anti-O-ag IgG (64,75). Moreover, IgM has been shown to better tolerate low antigen density than IgG, which may as well be attributed to IgM's high avidity (29). Concluding, the high avidity of IgM seems to be beneficial in targeting more variable antigens like CPS or LPS, which falls in line with its natural appearance as the most generic antibody in the immune response, or low-density antigens.

Furthermore, IgM has been observed to be more effective than IgG in activating complement in response to distal antigens (29). This might be due to the unique staple-like shape of surface bound IgM, which allows closer proximity of the C1-complex to the target membrane, enabling optimal C4b deposition, as discussed in chapter 6.2 (34). While the maximum distance from the membrane at which IgM can still activate complement remains to be explored, these structural advantages of IgM could be beneficial in targeting large antigens like CPS and LPS, which are typically located further away from the bacterial membrane. However, it may also be that at a certain distance to the membrane, complement activation will occur without inducing effective insertion of the MAC into the membrane and subsequent cell lysis. Consequently, the optimal and maximal distance of an antigen to the surface for IgM induced complement activation still remain to be elucidated, even though IgM seems to tolerate greater distance better than IgG (29).

IgG1 and IgG3 are both expressed as monomers and thus have a lower avidity than IgM (1). However, they have naturally higher affinity towards their antigen, seeing as they have undergone affinity maturation (85). This gives them distinct advantages when targeting more specific antigens but is also accompanied by a lower tolerance for variation. For instance, it has been shown that IgG antibodies targeting highly variable CPS have reduced oligomerisation capacity, which is crucial for their ability to activate complement (86). However, the increased affinity and smaller size of IgG antibodies may enable them to access and bind to more concealed and more specific antigens like OMPs (1,8). Interestingly, one study focusing on IgM and IgG1 complement activation against a specific antigen, StrepTagII genetically engineered onto an OMP in *E. coli*, found that between IgM and IgG1 antibodies of similar, high affinity, IgM was about 70-fold more efficient at inducing complement than IgG1(84). However, this was in a controlled setting with no antigen variation, where affinity is much more important than avidity. It might be interesting to investigate the balance between affinity and avidity across antigen targets with increasing variability. However, overall IgG seems to be better suited to target more specific antigens rather than generic ones, which falls in line with its natural occurrence further along the line of adaptive immunity than IgM (11).

Moreover, when directly comparing IgG1 and IgG3 antibodies in complement activation regarding their affinity, this reveals that IgG3 can probably maintain higher affinity and still activate complement effectively compared to IgG1. This is due to structural variations in their hinge regions. The more flexible hinge region of IgG3 allows for bivalent binding and simultaneous oligomerisation, resulting in higher avidity of the IgG3 Fc platform, while the more rigid hinge region of IgG1 favours oligomerisation when IgG1 is monovalently bound, as discussed in chapter 5.2.2 (35,47). If the affinity of IgG1 is too high, the probability of it to loosen one Fab binding to en-

gage into oligomerisation is less likely, which is why a lower affinity in IgG1 antibodies favours complement activation (87). This could implicate that IgG3 may be able to compensate for lower density or higher variable antigens compared to IgG1, which however remains to be tested. Additionally, the flexibility of the IgG3 hinge region also enables the formation of Fab arrays at high antigen densities, potentially leading to increased antibody coverage of the target and, consequently, increased effector functions (35). However, the elongated and flexible IgG3 hinge region is more susceptible to proteolytic cleavage, which probably contributes to its shorter half-life (1). Consequently, to fully exploit the advantages of IgG3 over IgG1 in complement activation at low antigen densities and regarding affinity for therapeutic purposes, there is dire need to increase IgG3 half-life and test its therapeutic potency compared to IgG1.

Additionally, IgG1 and IgG3 have a higher dependency on the density of their target antigens and proximity to the membrane compared to IgM (29,35). This means that some low-density antigens, which might be very specific for a certain bacterial strain, are not effectively targetable by IgG. One the one hand, this might be due to the limited distance that the Fab arms of a monomer can cover, which, due to its long and flexible hinge region, IgG3 can compensate better for than IgG1 (35). On the other hand, the need of monomeric IgG to oligomerise in order to induce complement activation poses a challenge during low antigen density, where fewer antibodies are available for oligomerisation (11,35). Here, IgG is also more susceptible to bacterial defence mechanisms preventing antibody oligomerisation than IgM, which already circulates as a multimer (8,88). Furthermore, the binding of more concealed antigens that are closer to the membrane may result in the Fc platform of IgG oligomers becoming obscured, thereby reducing their accessibility for interaction with the C1-complex or other receptors, as discussed in chapter 6.1 (35). This limitation could potentially impact the efficacy of IgG in engaging the complement system or other effector functions. Taken together, the limitations of IgG1 and IgG3 antibodies regarding antigen density and distance to the membrane should be carefully considered in mAb design and the extend of those limitations should be investigated further.

In conclusion, each antibody isotype possesses distinct characteristics that theoretically render them suitable for targeting specific antigens. However, the identification of these optimal targets requires further investigation, particularly through bacterial studies. While controlled experimental settings can provide fundamental knowledge, the insights gained must be validated and tested in *in vivo* experiments. While natural immunity has optimised the usage of different antibody isotypes for different aims, there is a need to further understand and apply this knowledge to monoclonal antibody therapies, especially in the context of growing antibiotic resistance.

9 Conclusion

The efficacy of antibody-mediated complement activation on bacteria is influenced by a multitude of factors. Key determinants include structural differences among antibody isotypes and subclasses, which substantially influence their binding potential and capacity to interact with proteins such as other antibodies and the complement component C1q. While for example the high Fc platform of IgG3 allows for increased accessibility for C1q, the Fc platform is also less rigid which might result in less binding affinity between C1q and IgG3. Furthermore, the efficacy of these antibodies is significantly influenced by the characteristics of their target antigens, particularly factors such as antigen availability, spatial positioning, and proximity to the bacterial membrane. For instance, the high avidity of IgM allows it to bind more variable or distal antigens, while IgG can target more specific antigens and antigens that are

less accessible. In recent years, the knowledge surrounding antibody characteristics, particularly in relation to bacterial infections, has increased substantially. Despite this progress, there is still a dire need for further research to elucidate the optimal pairing of specific antibodies with corresponding bacterial antigens. Here, a focus on bacterial studies to enhance our understanding of the immune response against bacterial pathogens is of particular interest. Such insights are critical for the development and refinement of future (monoclonal) antibody therapies, particularly in the context of increasing antibiotic resistance.

10 Laymen Summary

In this review, we look closely at the role of different types of antibodies in fighting off bacterial infections. More specifically, we are focusing on how antibodies help activate a part of the immune system called the complement system. This system is a group of proteins that work together to attack and kill bacteria and other pathogens. Antibodies can be divided into five different groups called isotypes. We focused on the isotypes IgG and IgM because they are the best at activating the complement system. IgM is a larger protein which is made up of five or six single parts and takes on the shape of a pentagon or hexagon. This shape allows it to bind strongly to multiple parts of a bacterium, which means that it can hold onto the bacterium very well. This makes it a potent and important part of the antibacterial immune response. However, its large structure can also make it hard for IgM to reach some parts of the bacteria and it can more easily be blocked. IgG is a smaller protein only made up from a single part and can consequently reach some parts of the bacterium better than IgM. Moreover, IgG antibodies have undergone a special process that fine-tuned them for a specific target, allowing them to bind more precisely to a bacterium. There are four different subclasses of IgG antibodies, of which IgG1 and IgG3 are the most potent at activating the complement system. The most interesting difference between them is the so-called hinge region, which determines how flexible and bendable the antibody is, much like an elastic coil spring. This influences their size and ability to bind to the bacterium, which in turn has great influence on their ability to activate the complement system. Based on their characteristics, different antibodies are better suited to target certain parts of the bacteria than other antibodies. Here, factors like the size of the antibody target, also called antigen, and its distance to the surface of the bacteria play a role. If the antigen is too far from the surface of the bacteria, it can be difficult for the antibody to properly activate the complement system. Furthermore, an antigen needs to be present in high enough numbers for the antibodies to bind them properly. For example, IgM can better target parts of the bacteria that are present in less high numbers due to its large size and multiple parts, while IgG1 and IgG3 need their target to be present in higher numbers since they are composed of only one part. Overall, it is very important to understand how different antibodies interact with the complement system to fight of bacteria, in order to make use of this in antibacterial therapy.

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