Clustering: a rational design principle for potentiated antibody therapeutics

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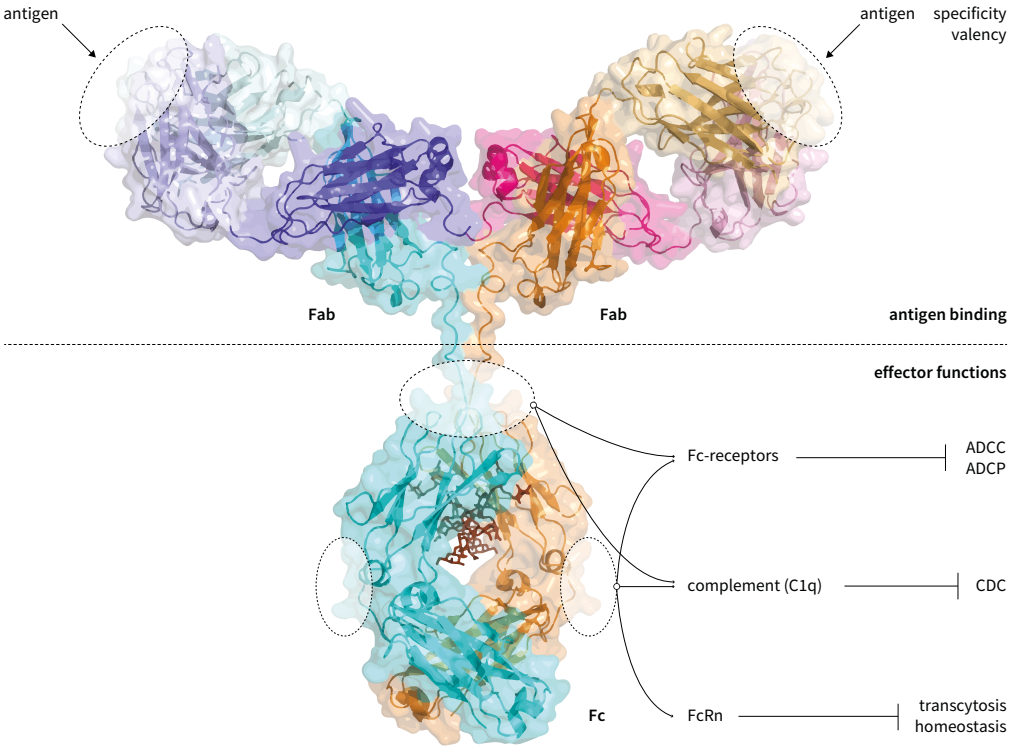
AIM AND OUTLINE OF THIS THESIS

For centuries, the field of medicine was dominated by the belief in a Greek theory suggesting that all disease was caused by an imbalance of the four 'humors': black bile, yellow bile, phlegm and blood. It was not until the 19th century that this view was challenged by Ilya Metchnikoff, Emil Behring and Shibasaburo Kitasato[2]. Metchnikoff discovered that phagocytic cells could respond to injury in starfish and other invertebrates, while Behring and Kitasato reported that something in the blood of mice immunized with diphtheria and tetanus toxins seemed to mediate antitoxic activity. These observations shifted the paradigm towards a separation of the immune system into two branches: cellular immunity, for which protection is mediated by cells and humoral immunity, for which protection is mediated by substances in the humors, or body fluids. The substances present in the humors were later identified as antibodies produced by B lymphocytes, amongst other macromolecules such as complement components.

In humans, there are five classes of antibodies, or immunoglobulins (Ig) – IgA, IgD, IgE, IgG and IgM, of which IgG is the most abundant comprising of up to 75% of serum Ig. IgG, consisting of four subclasses (IgG1-4), is the main immunoglobulin class used as therapeutic agent. The general structure of IgG molecules is similar amongst subclasses, each consisting of a Fab domain with two Fab arms and an Fc domain (Figure 1). Structural differences between subclasse§s are mainly found in the hinge region, which confers the flexibility of the molecule, and accounts for a large part of the variation in effector function activation observed between subclasses. The Fab domain is responsible for highly specific antigen recognition and consists of two Fab-arms with identical binding sites that may bind simultaneously (bivalent) or alternatingly (monovalent) to target antigen(s). The Fc domain is involved in binding complement or Fc receptors (FcRs) present on immune cells and is



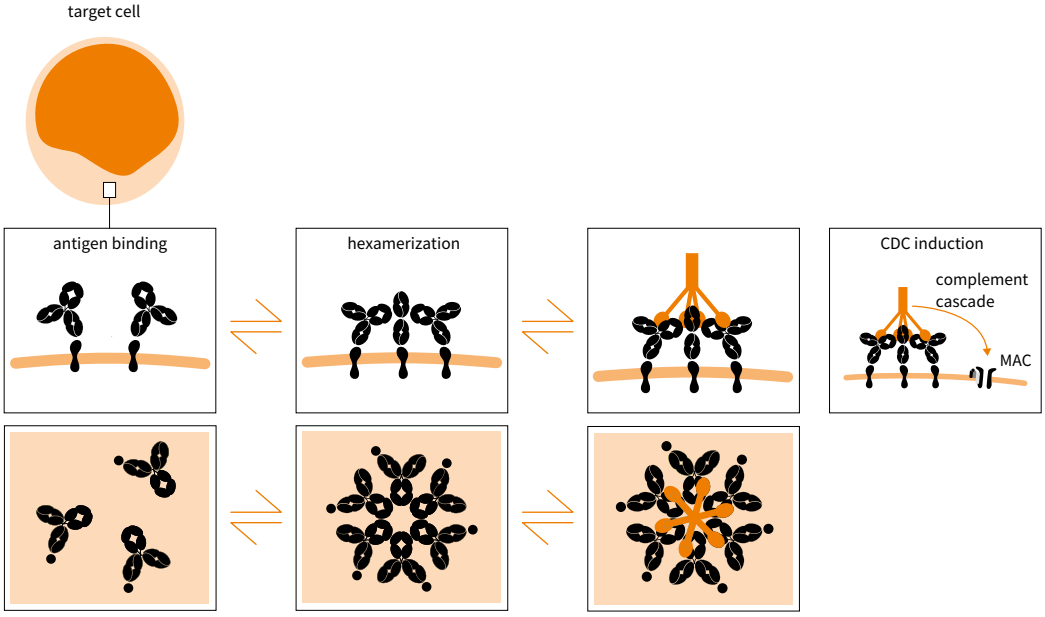
▲ **Figure 1**

IgG antibody structural and functional characteristics.

thereby capable of activating a variety of effector functions including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). The Fc-region also contains a binding site for the neonatal Fc receptor (FcRn) that regulates serum half-life of IgG through transcytosis, or bidirectional transport across polarized cellular membranes.

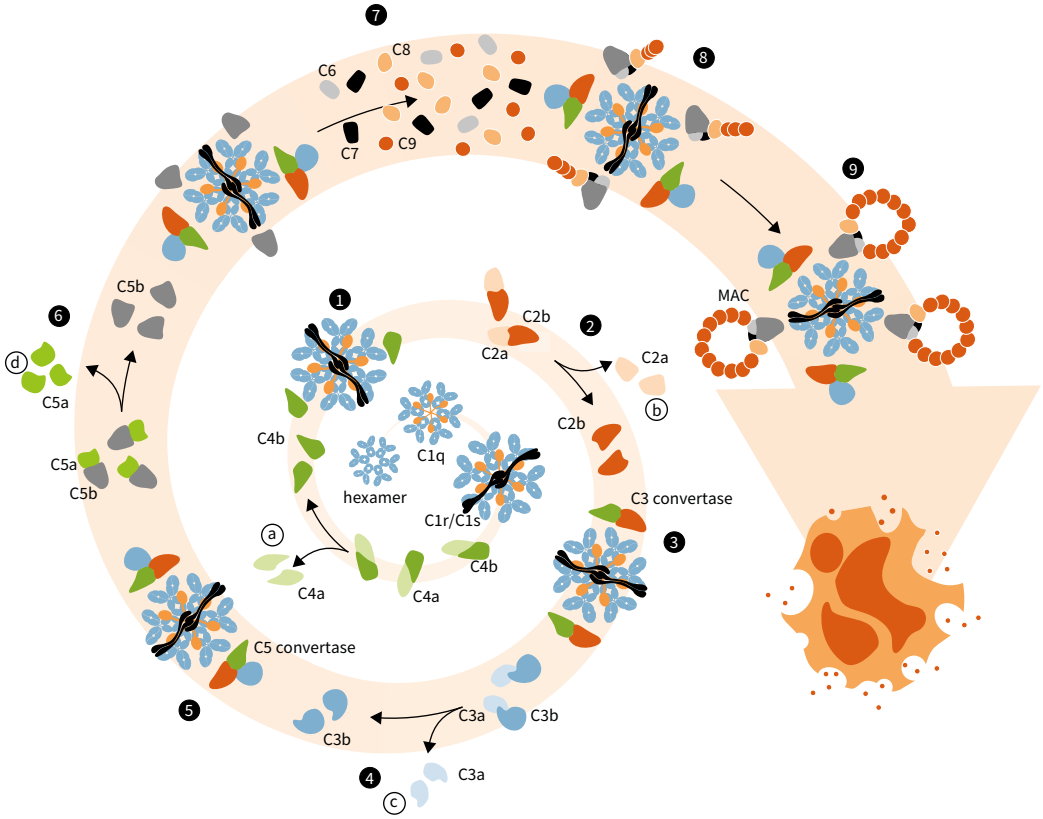
Due to their high specificity and capacity to initiate a range of effector functions, antibodies are widely used as therapeutics for treatment of cancer, infectious diseases and autoimmunity. Antibody-based therapy evolved from passive immunization using polyclonal antisera to the discovery the hybridoma technique that allowed for the stable and controlled generation of monoclonal antibodies (mAbs) of single origin and specificity[3,4]. Although the success of mAb-based therapeutics is illustrated by over 100 antibodies having been granted marketing approval to date, disease heterogeneity and plasticity drive resistance to targeted therapies, demonstrating the continuous need for novel and more efficacious drugs[5,6]. A key feature shared by many high-

ly efficacious therapeutic antibodies including, amongst others, rituximab, ofatumumab, daratumumab, cetuximab and trastuzumab, is their capacity to engage the immune system and initiate effector functions. It is generally acknowledged that a single binding interaction between an antibody and antigen is generally not sufficient to successfully activate effector functions. Instead, antibody-mediated effector function activation often requires multivalent target binding and cell surface clustering to effectively recruit immune effector molecules or cells of the immune system. For example, ADCC and ADCP are initiated through crosslinking of clustered antibody Fc domains with Fc gamma receptors (FcγRs) expressed on immune effector cells such as natural killer (NK) cells, macrophages and neutrophils. Furthermore, while it has long been recognized that membrane-bound antibodies or immune complexes can induce CDC through the so-called classical complement pathway, it was discovered more recently that this pathway is optimally activated by cell-bound IgGs organized into ordered hexameric clusters through non-covalent Fc-Fc interactions (Figure 2)[7]. Hexameric IgG clusters efficiently bind and activate complement factor C1, consisting of a hexavalent C1q subunit and associated proteases C1r and C1s, thereby triggering a series of enzymatic events at the cell surface that ultimately kill the cell (Figure 3)[8].



▲ **Figure 2**

The biology of IgG hexamerization and complement activation.
Cell-bound IgGs organize into ordered hexameric clusters through non-covalent Fc-Fc interactions, thereby facilitating efficient C1q binding and activation of the complement cascade. Complement activation results in induction of complement-dependent cytotoxicity (CDC) through the formation of the membrane attack complex (MAC). Upper panels: side view, lower panels: top view.



▲ **Figure 3**

Schematic overview of the classical complement pathway.
Cell-bound IgGs organize into ordered hexameric clusters, thereby providing an optimal docking site for the hexavalent C1q subunit and associated proteases C1r and C1s that together form C1 complex. (**1**-**4**) Activated C1 complex cleaves C4 and C2 into fragments to form C3 convertase (C4b2b - according to old terminology, C2a and C4b2a[1]), which subsequently cleaves complement component C3 into C3a and C3b. (**5**) High density cell surface deposition of C3b results in association of C3b with existing C3 convertase to form C5 convertase (C4b3b2b). (**6**-**8**) Proteolysis of C5 by C5 convertase produces C5b, which triggers the terminal phase of complement activation by binding C6, C7, C8 and multiple copies of C9. (**9**) C5b-9 together form lytic pores in the cell membrane termed membrane attack complexes (MACs), which eventually kill the cell. (**A**-**D**) Proteolysis of complement components throughout the cascade generates small byproducts (anaphylatoxins) with potent chemoattractant properties, and opsonins that recruit and activate immune effector cells.

In natural biology, an effective immune response often involves multiple distinct antibodies originating from multiple B-cell clones, also referred to as polyclonal antibodies, which all bind specific structures, or antigens expressed on the pathogenic target. Importantly, a polyclonal antibody response often yields antibodies having the capacity to recognize multiple (non-overlapping) epitopes on a single antigen, thereby allowing for sufficient target occupancy and high local density of IgG Fc domains to enable clustering-dependent initiation of effector functions. Polyclonal antibodies therefore provide an advantage to the immune system compared to mAbs, which are of single origin and react against a single antigen or antigenic epitope. The single

antigen or epitope specificity of mAbs can strongly influence effector function activation. For example, many mAbs have a weak intrinsic ability to trigger the complement cascade and induce CDC. Multiple studies have shown that the lack of CDC activity by mAbs can be overcome by combining mAbs that recognize non-overlapping epitopes on a single antigen, suggesting that a certain level of ordered clustering via self-assembling Ig Fc domains is required prior to activation[9-14].

The importance of antibody clustering in driving efficient effector function activation is becoming increasingly recognized, as is demonstrated by a broad spectrum of novel engineering strategies that leverage antibody clustering mechanisms to boost 'classical' as well as novel or 'designed' effector functions. In the context of 'classical' effector functions, antibody-mediated CDC can be improved by single point mutations in the IgG Fc domain that increase intermolecular Fc-Fc interactions upon binding to membrane-bound targets, thereby facilitating enhanced IgG hexamer formation and C1q binding[7,15]. Moreover, Fc domain engineering may also promote novel or 'designed' effector functions typically not obtainable using conventional mAbs. Enhancing IgG hexamerization was shown to allow the possibility of using monoclonal antibodies to induce hyper clustering of tumor necrosis factor receptor superfamily (TNFRSF) cell surface receptors including death receptor 5 (DR5) and OX40, resulting in increased agonistic activity[11,16]. Such engineering approaches illustrate the relevance of antibody clustering mechanisms in efficient effector function activation. The aim of this thesis was to further explore the role of antibody clustering in the mechanisms of action and design of effective antibody-based therapeutics. This research provides insight into the importance of 'ordered clustering' in antibody function and how this knowledge may directly translate into novel antibody-based therapeutics.

Previous research studying the molecular events governing complement activation demonstrated that antibody clustering or hexamerization after target binding to the cell surface is essential for optimal binding of C1q and efficient activation of the proteolytic cascade of complement[7]. In **Chapter 2**, we expanded on this observation and addressed the question whether multiple antibodies targeting different cell surface receptors are capable of cooperatively engaging in complement activation by forming mixed (hetero-) hexameric clusters on the cell surface.

Further building on the knowledge that enhancing intermolecular Fc-Fc interactions through single point mutations, such as E430G, in the Fc domain can increase antibody hexamerization and complement activation[15], led us to explore the use of such mutations for the design of novel therapeutic antibodies with enhanced potency. In **Chapter 3**, we describe the generation and

characterization of DuoHexaBody-CD37, a novel CD37-targeting antibody for the treatment of B-cell malignancies, which potently activates complement through a combination of dual epitope targeting and enhanced hexamerization. The preclinical activity of this antibody molecule was further studied *ex vivo* in primary tumor cells derived from patients with various B-cell malignancies, as described in **Chapter 4**. The potent preclinical anti-tumor activity observed *in vitro*, *ex vivo* and *in vivo* in a broad spectrum of B-cell malignancies provided the preclinical rationale to advance this molecule into clinical development and initiate a first-in-human clinical trial (NCT04358458).

Based on the observation that antibodies targeting different membrane receptors can hetero-oligomerize into mixed hexameric complexes upon antigen binding (**Chapter 2** of this thesis), we further explored whether IgG antibody pairs could be engineered to act as Boolean logic AND gates selectively activated after hetero-oligomerization in **Chapter 5**. Logic AND gates, originating from electrical engineering, can convert a combination of input signals into outputs according to the laws of Boolean algebra. Fc-domain engineered IgG antibody pairs were designed to integrate two antibody binding signals into a functional response output only on cells or surfaces co-expressing both antibody targets, thereby enabling the creation of antibody-based therapeutics with improved safety and efficacy.

Understanding the relationship between antibody structure and function and more specifically, how antibodies interact with their target, has proven to be essential in the design of next-generation antibody-based therapeutics. The crucial role of antibody (-mediated) clustering in successful effector function activation has become more apparent in recent years. In **Chapter 6** we reviewed (therapeutic) antibody effector mechanisms, with particular emphasis how antibody (-mediated) clustering impacts functional response. We further described how tuning of antibody clustering can serve as a design basis for engineering to increase the functional activity of novel antibody-based therapeutics and provided an overview of current translational efforts regarding clustering-based antibody concepts in the clinic.

Finally, a general discussion summarizing the key findings of this thesis is provided in **Chapter 7**. The relevance of these findings are discussed in the context of the past, present and future landscape of antibody-based therapeutics.

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