Clustering: a rational design principle for potentiated antibody therapeutics

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GENERAL DISCUSSION

Antibodies represent a class of therapeutic agents that revolutionized the development of 'targeted therapies' against disease. In contrast to most small molecule drugs, which often are more prone to off-target adverse effects, antibodies are highly target-specific and capable of efficiently interacting with the host immune system. Owing to their modular and highly adaptable architecture, relatively predictable developability and favorable pharmacokinetics, they are also highly suitable for engineering into formats with novel functionalities, improved drug safety or increased potency. Nevertheless, optimally designing and tuning effector functions towards a desired outcome and 'improving on nature' by mimicking the full potential of antibody functional activity as observed in natural immunity is not straightforward. Understanding the key determinants that govern efficient effector function activation is thereby crucial in the design of novel and more efficacious antibody-based therapies. One of the factors central to efficient effector function activation is the accumulated strength, or 'avidity' of binding interactions between antibodies and their target (**Chapter 6**). The aim of this thesis was to explore the role of antibody avidity interactions, and more specifically the importance of 'ordered clustering', in antibody mechanisms of action and to apply the knowledge obtained in designing novel and improved antibody-based therapeutics.

Enhancing therapeutic antibody function: (hetero-) hexamerization

Antibody-mediated activation of the classical complement pathway represents a powerful effector mechanism that triggers killing of the target cell via complement-dependent cytotocixity (CDC). Moreover, intermediates in the complement cascade can act as anaphylatoxins with potent chemoattractant properties, and opsonins that recruit and activate immune effector cells[1]. Complement is activated through binding of C1q to antigen-bound IgM or IgG antibodies. High avidity binding of C1q to IgG molecules requires oligomerization of neighboring IgG Fc regions into ordered hexameric arrangements via specific non-covalent Fc-Fc interactions[2]. Many monoclonal IgG antibodies are insufficiently capable of

hexamerization and CDC induction, which relates to multiple factors *inter alia* including target biology, target density, epitope specificity and overcoming target cell complement protection and resistance. It is now demonstrated that monoclonal antibodies (mAbs) targeting two different cell surface antigens may cooperate in complement activation through the formation of hetero-hexamers (**Chapter 2**). In addition to CD20, CD37 is abundantly expressed on malignant B cells and membrane-bound CD20 and CD37 antibodies were shown to colocalize by forming mixed hexameric complexes and synergize in CDC activity. Previously, it was demonstrated that antibody hexamer formation and complement activation could be increased by introducing specific point mutations, such as E430G and E345K, in the Fc domain[3]. These mutant antibody variants potentiated or enhanced CDC of tumor cells expressing a range of different target antigens including CD20, CD38, CD52 and EGFR. Likewise, while wild-type (WT) IgG mAbs targeting CD37 are poor inducers of CDC, introduction of the E430G hexamerization-enhancing mutation substantially potentiated CDC of malignant B cells (**Chapter 2 & 3**). In addition, we showed that the E430G mutation further increased antibody colocalization, hetero-hexamerization and complement activation by combinations of CD20 and CD37 mAbs (**Chapter 2**). These results may have clinical relevance for combination therapies, as enhancing antibody Fc-Fc interactions to bring different cell surface receptors in close proximity could potentially broaden the combinatorial therapeutic target space. In this respect, understanding antibody structural characteristics in combination with target receptor biology and distribution are key in the development of effective antibody combination therapies. How different antibody formats may impact function is discussed in the next paragraphs.

Enhancing therapeutic antibody function: the impact of different Fc formats

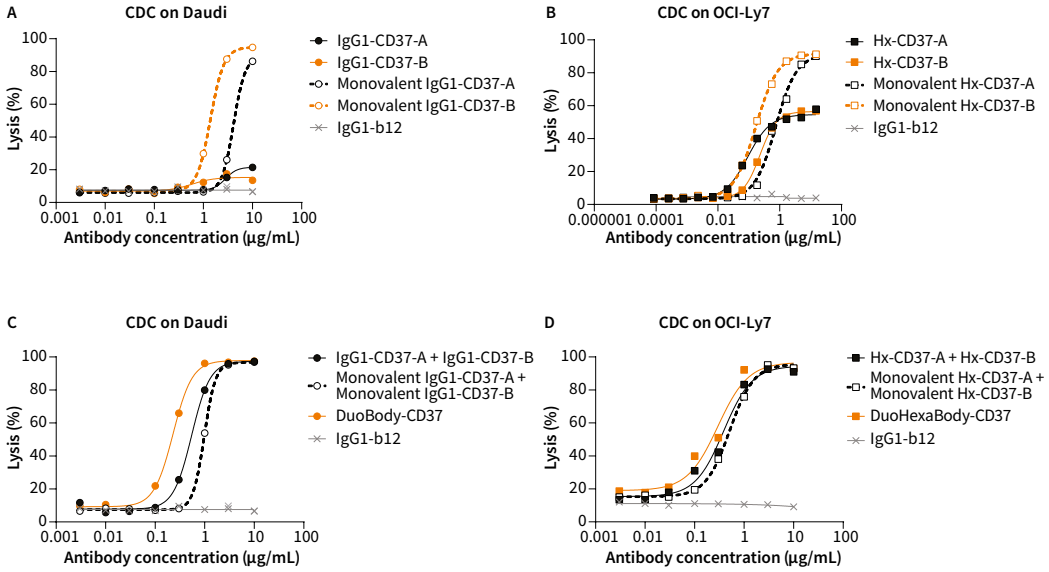
In cancer, complement activation has been demonstrated to contribute to the tumor cell kill mediated by therapeutic antibodies targeting various cell surface receptors expressed on hematologic tumor cells including CD20, CD38 and CD52[4-6]. Different CD20-targeting antibodies have been developed for treatment of B-cell malignancies including rituximab, ofatumumab and obinutuzumab, all of which vary in their capacity to activate complement. CD20 antibodies are generally classified as type I or type II, each type exhibiting distinct binding and functional characteristics[7]. Type I CD20 antibodies including rituximab and ofatumumab bind CD20 dimers in a 2:2 stoichiometry (2 Fabs per CD20 dimer)[8,9]. This antibody binding orientation may lead to rapid CD20 concatenation and antibody hexamerization, thereby facilitating efficient complement activation[10]. The next-generation CD20 antibody ofatumumab activates complement more effectively than rituximab by binding a CD20 epitope located closer to the cell membrane, thereby allowing more efficient C1q binding and complement deposition[11]. By contrast, the type II CD20 antibody

obinutuzumab only weakly activates complement, presumably due to binding CD20 in a 2:1 stoichiometry (1 Fab per dimer) that results in steric hindrance between Fabs and a decreased ability to form hexameric complexes[8,9].

Targeting of alternative B-cell antigens has also been explored, including the tetraspanin plasma membrane protein CD37. Like CD20, CD37 is mainly expressed on mature B cells and B cell-derived malignancies and represents an attractive target for novel antibody therapies[12-15]. While to date, multiple CD37-targeting antibodies have been described, none of these reported potent activation of the complement cascade[16]. Based on our previous observation that CDC by CD37 antibodies could be potentiated by enhanced antibody clustering via a single point mutation E430G in the IgG Fc domain (**Chapter 2**), I sought to investigate the therapeutic potential of this antibody technology platform. In **Chapter 3**, the generation and characterization of a panel of CD37 antibodies containing an E430G mutation in the Fc domain is described. The mutation was added in an effort to optimize CD37 antibodies to engage in multiple effector mechanisms, specifically potent CDC. During lead candidate selection, multiple CD37 antibody clones exhibiting potent CDC activity were identified, some of which were able to bind CD37 simultaneously despite the relatively small extracellular domain (151 amino acids). Efficient CDC induction depends on locally increasing the density of IgG Fc domains, thereby facilitating enhanced Fc-Fc interactions and efficient antibody hexamerization. In that respect, increasing CD37 target binding by combining non-crossblocking CD37 antibodies, either as antibody mixtures or in a biparatopic (bispecific) configuration, appeared a promising approach to further enhance CDC. Therefore as a next step, single CD37 antibodies, combinations of non-crossblocking CD37 antibodies and biparatopic (bispecific) CD37 antibody variants were systematically evaluated for their CDC-inducing capacity. Surprisingly, the biparatopic (bispecific) CD37 antibody variant, DuoHexaBody-CD37, outperformed all other antibody variants evaluated both *in vitro* and *ex vivo*. This superior CDC activity was attributed to a combination of dual epitope targeting and enhanced antibody hexamerization in the context of the E430G Fc mutation.

The superior CDC efficacy of the biparatopic (bispecific) CD37 antibody compared to a combination of two non-crossblocking antibodies, especially in relapsed/refractory primary chronic lymphocytic leukemia (CLL) patient samples, raised questions about the binding mechanism of the bispecific and whether hexamer formation could be favored in certain binding configurations. Cryo-electron tomography illustrated that the optimal docking platform for C1q consists of six monovalently-bound antibodies arranged into a hexameric complex through Fc-Fc interactions[2]. I therefore hypothesized a preferred role for monovalent antibody binding in antibody hexamer formation and

tested different WT or hexamerization-enhanced (Hx) functionally monovalent CD37 antibody variants (one CD37-binding Fab-arm and one non-binding IgG-b12 Fab-arm in a bispecific configuration) and combinations thereof in CDC assays. Interestingly, functionally monovalent variants of both WT (IgG1-) and HexaBody (Hx-) CD37 antibodies showed enhanced CDC activity compared to their bivalent counterparts (Figure 1 A-B), suggesting that monovalent binding enables more efficient Fc-Fc-mediated hexamerization and CDC. Potentiated CDC activity has previously been reported for functionally monovalent bispecific antibody variants targeting EGFR on solid tumor cells[2]. In experiments comparing combinations of IgG1- or Hx-CD37 antibodies targeting non-overlapping epitopes, either bivalent or functionally monovalent, CDC potency was highly comparable (Figure 1 C-D). Minor differences in EC50 values between functionally monovalent mixtures versus bivalent antibody mixtures are most likely caused by the availability of CD37 binding arms. Furthermore, IgG1 and Hx biparatopic (bispecific) antibody variants (bsAb-CD37 and DuoHexaBody-CD37 respectively) showed at least comparable or increased CDC activity compared to the respective bivalent or monovalent binding CD37 antibody combinations. Overall, monovalent rather than bivalent antibody binding appears to contribute to enhanced CDC potency, both in the context of IgG1- and Hx-CD37 antibody variants. In case of bivalent CD37 antibody combinations targeting non-overlapping CD37 epitopes, I cannot exclude that these also drive monovalent binding. Strasser et al. recently reported that bivalent-binding antibodies might sterically suppress Fc-Fc interactions occurring through lateral diffusion across cell surfaces[17]. In IgG1- or Hx-CD37 antibody combinations targeting non-overlapping CD37 epitopes, binding competition may also sterically suppress bivalent antibody binding and conversely promote monovalent antibody binding and lateral diffusion. Here, the interplay between antibody affinity and multiple levels of avidity interactions are key determinants of the functional outcome (**Chapter 6**). Antibodies with higher affinities (i.e. bivalent) have slower off rates, which promote avidity interactions through multivalent antibody binding, but may thwart the assembly into higher order avidity structures. Conversely, lower affinity (i.e. monovalent) antibodies have faster off rates that may preclude multivalent binding interactions. They can however, promote higher order avidity structures through efficient Fc-Fc interactions, resulting in the formation hexameric complexes that provide sufficient avidity for subsequent C1q binding. Similarly, mimicking polyclonal antibody binding through multi epitope targeting may add an additional level of complexity to avidity interactions and the resulting functional response. Thus, optimally balancing multiple levels of antibody avidity including 1) binding or first order avidity (monovalent versus bivalent interactions), 2) higher order avidity (functional interactions with effector molecules) and 3) cooperative avidity (result of polyclonal binding on



▲ **Figure 1**

Effect of monovalent binding, enhanced hexamerization and dual epitope targeting on the CDC activity of CD37 antibodies.

CD37-A and CD37-B represent different humanized CD37 antibody clones (010 and 016 respectively) binding non-overlapping epitopes on CD37. Functionally monovalent variants of both WT IgG1 and Hx-CD37 antibodies were generated through controlled Fab-arm exchange with a non-binding IgG1-ctrl arm (IgG1-b12). IgG1-b12 served as a non-binding control antibody. Daudi and OCI-Ly7 cells express a mean of 227.832 and 99.654 CD37 copies/cell respectively. (**A**) Comparing CDC activity between bivalent wild-type (WT) IgG1-CD37 and functionally monovalent IgG1-CD37 antibody variants on Daudi cells. (**B**) Comparing CDC activity between bivalent hexamerization-enhanced (Hx)-CD37 and functionally monovalent Hx-CD37 antibody variants on OCI-Ly7 cells. (**C**) Comparing CDC activity among combinations of bivalent WT IgG1-CD37 antibodies, combinations of functionally monovalent WT IgG1-CD37 antibodies and biparatopic (bispecific) WT IgG1-CD37 antibodies (BsAb-CD37) expressed on Daudi cells. (**D**) Comparing CDC activity among combinations of bivalent Hx-CD37 antibodies, combinations of functionally monovalent Hx-CD37 antibodies and biparatopic (bispecific) Hx-CD37 antibodies (DuoHexaBody-CD37) expressed on OCI-Ly7 cells. Data shown are representative of three repeat experiments.

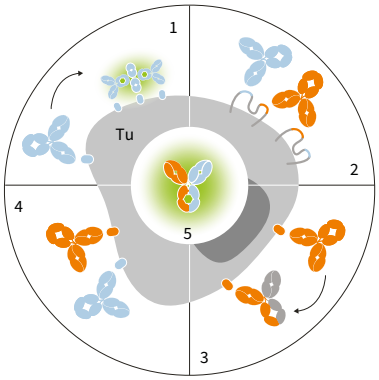
functional interactions), is crucial in understanding antibody mechanisms of action and designing more effective antibody therapeutics.

In conclusion, the superior CDC activity observed for DuoHexaBody-CD37 may be driven by efficient hexamerization facilitated by monovalent binding of the Fab arms to distinct CD37 epitopes (i.e. two antibody molecules monovalently-bound to one CD37 antigen), which is further enhanced through the E430G mutation. Monovalent antibody binding potentially driving IgG oligomerization and effector function activation, by optimally balancing affinity and avidity interactions, is an important observation with wider implications for antibody-based drug discovery.

The unique CDC-inducing capacity and ex vivo therapeutic potential of DuoHexaBody-CD37 was further evaluated in **Chapter 4**, in which potent complement-mediated tumor cell kill was observed in primary CLL and B cell non-Hodgkin's lymphoma patient samples irrespective of the patients' relapse status, prior treatments or malignancy subtype. Additionally, and consistent with observations in **Chapter 2**, combining DuoHexaBody-CD37 with rituximab or ofatumumab significantly enhanced tumor cell kill in patient samples with intermediate to low sensitivity to DuoHexaBody-CD37-mediated CDC. Notably, CD37 and CD20 expression levels substantially differed in experiments performed using tumor cell lines (**Chapter 2** and **Chapter 3**) versus experiments performed using primary patient cells (**Chapter 4**). In cell lines, DuoHexaBody-CD37-mediated CDC was generally most potent at CD37 expression levels above 100,000 molecules/cell. By contrast, the CD37 expression threshold for DuoHexaBody-CD37-mediated CDC was substantially lower in primary patient cells that expressed a median of 35,000 molecules/cell. The superior potency of DuoHexaBody-CD37 was also more prominent in primary patient cells rather than tumor cell lines, which may be attributed to differences in complement regulatory protein levels, receptor density, cell size, or the spatial organization of CD37 and associated proteins in tetraspanin-enriched micro domains in the cell membrane[10,18,19]. Furthermore, WT IgG1 antibodies generally require higher antigen expression levels for effective initiation of CDC[17,20,21]. These data suggests that multiple antibody engineering strategies including 1) enhancing IgG Fc-Fc interactions through the E430G mutation, 2) dual-epitope targeting, 3) monovalent antibody binding and 4) targeting multiple cell surface antigens or (5) a combination of these strategies, may enhance antibody and/or target clustering and thereby lower the threshold for complement activation (Figure 2). DuoHexaBody-CD37 uniquely combines the E430G Fc mutation and dual epitope targeting in a biparatopic (bispecific) antibody to potentiate CDC as an additional effector mechanism and illustrates how systematic evaluation of different antibody format technologies may positively impact the selection of lead compounds with broader mechanism of action and therapeutic function. The potent anti-tumor activity observed *in vitro*, *ex vivo* and *in vivo* provided the rationale to initiate a phase 1/2 first-in-human clinical trial to assess the clinical safety and preliminary efficacy of DuoHexaBody-CD37 in patients with hematologic malignancies (NCT04358458).

Enhancing therapeutic antibody function: ordered clustering as a tool for modular therapeutic design

Despite their modular and highly adaptable architecture, the vast majority of current antibody engineering strategies are aimed at designing antibody therapeutics acting as single molecular entities. As reviewed in **Chapter 6**, the polyclonality of natural antibody immune responses that effectively exploit



◄ Figure 2

Engineering strategies to enhance antibody (-mediated) clustering and complement activation.

1) Enhancing IgG hexamerization using an E430G Fc mutation. 2) Dual-epitope targeting. 3) Monovalent antibody binding. 4) Targeting multiple cell surface antigens. (5) A combination of aforementioned engineering strategies, here illustrated as a biparatopic/dual-targeting hexamerization-enhanced antibody molecule.

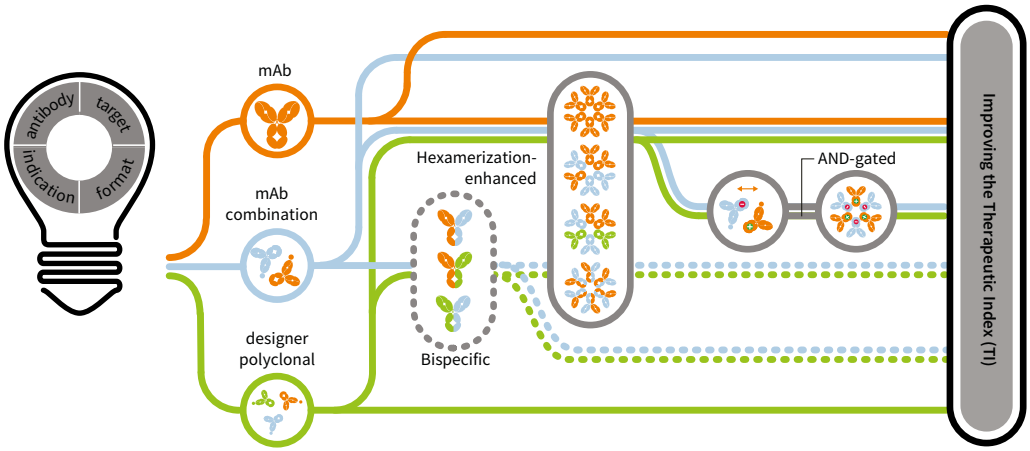
immune complexation by conjoining multiple affinities, valencies and binding specificities, argues for more effort towards rational design of combinatorial therapeutic approaches. Rational designer polyclonals for instance, represent a next frontier for therapeutic development. In **Chapter 2**, the therapeutic potential of such combinatorial approaches was illustrated by IgGs targeting different cell surface antigens that co-engaged in mixed hexameric complexes through non-covalent interactions between Fc domains. These observations laid the foundation for a novel antibody technology based on minimally engineered antibody combinations with molecular Fc interfaces designed to assemble into teams only if both components are bound to the same cell (**Chapter 5**). It is shown how IgG hetero-oligomerization and functional activation can be made dependent on the presence of two antibody components by introducing specific point mutations to modulate Fc-Fc interactions between these two components, C1q and Fc gamma receptor interactions. By requiring a combination of two input signals to license activation of a functional output signal, these antibody pairs act as a biologic equivalent of Boolean logic AND gates that allow for precisely tuning and restricting IgG avidity interactions and subsequent complex formation to preferred cell surfaces. Furthermore, this AND-gated approach may access a broader range of tumor or immune surface target molecules, of which expression is not sufficiently differentiated from healthy cells. A major challenge for potential therapeutic applications of mutually dependent antibody combinations is identifying cell surface receptors that co-localize and/or allow for antibody hetero-hexamerization after target binding. Compatibility with antibody hetero-hexamer formation is heavily influenced by individual target biological constraints including size, epitope, abundance, density, mobility, epitope-membrane distance and spatial organization[9,11,20,22]. While successful development of therapeutic antibodies relies on a thorough understanding of antibody and target biology, it is not trivial to predict which combination of antibody and format yields the most successful antibody drug candidate. The ‘plug and play’ nature of the Fc mutations that

form the basis of this antibody technology are readily applicable to combinatorial, high throughput screening of novel antibody target combinations to support drug development.

Perspectives

As discussed in this thesis and reviewed in **Chapter 6**, efficient triggering of antibody effector functions requires multivalent target binding and clustering of IgG molecules on the cell surface. Clustering of IgG molecules may provide an 'avid docking surface' that serves as a threshold for the subsequent binding and activation of immune effector molecules. In natural biology, antibody immune responses are polyclonal and highly efficient at establishing avidity interactions through the generation of antibodies with different affinities, valencies and (multi-epitope) binding specificities. A broad spectrum of novel antibody engineering strategies and formats are emerging that exploit antibody clustering mechanisms to optimally engage 'classical' effector functions including activation of complement and Fc receptors and enable novel therapeutic mechanisms[23-26]. Engineering strategies focusing on some of the strengths offered by polyclonals, including valency tuning via enhanced IgG oligomerization or creating functionally monovalent antibodies and tuning binding specificities by targeting multiple cell surface receptors or epitopes, may allow for more precisely coordinating and potentiating antibody functional responses. It is additionally demonstrated that distinct avidity engineering approaches might be combined to optimally engage antibody effector functions to achieve 'incremental' avidity effects. Nonetheless, the development of increasingly complex antibody architectures and combinatorial designs requires close monitoring of the balance between efficacy and safety, also referred to as the therapeutic index (TI). The TI for antibodies exploiting classical effector functions including rituximab, ofatumumab, obinutuzumab and daratumumab is often broad and relatively well established, however the effect of potentiating such effector functions on the TI is not always known. The recently initiated first-in-human trial for DuoHexaBody-CD37 may provide novel insights into how effector function potentiation impacts the TI. By contrast, the TI is often small and less well characterized for increasingly complex antibody formats including CD3 bispecifics and antibody-drug conjugates, as well as antibodies directed towards immuno-oncology targets such as checkpoint inhibitors. Improving the TI by optimizing on-target efficacy and/or decreasing off-target toxicity may broaden the applicability of such antibody formats. Optimizing the antibody TI however, is challenging and requires a thorough understanding of the corresponding disease biology, its underlying mechanisms and targets, combined with antibody format characteristics. These factors are all regarded as critical components for transformative antibody therapeutics and emphasize the importance of studying antibody structure-function relations and antibody interactions with their antigen to

move product ideation. In this thesis, it was demonstrated that exploiting antibody clustering through Fc engineering and/or multi-targeting approaches represents a promising strategy to enhance antibody efficacy and/or decrease off-target toxicity. These different approaches have been summarized and presented as a road map for next-generation drug development in **Figure 3**. Improving the understanding of which type of targets and effector functions can be exploited by antibody clustering, as studied in this thesis, may pave the way for a new generation of improved antibody-based drugs for treating and curing human diseases.



▲ **Figure 3**

Road map for next-generation antibody therapeutics.
The basis of antibody drug discovery starts with a rational idea bringing disease biology, targets and antibody format/backbone together. Improving the window between efficacy and/or safety of antibody drugs, also referred to as the therapeutic index (TI), requires engineering beyond the classical mAb format towards combinations or designer polyclonals and transforming the antibody backbone into a fit for purpose design. Different antibody engineering strategies discussed in this thesis are illustrated as stations within the road map leading towards improving and tailoring antibody function. Biologically focused drug discovery processes are crucial for the innovation of novel platform technologies that may broaden the road map towards the next generation of differentiated antibody therapeutics.

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