CD20 as target for immunotherapy

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General outline and aim of the thesis

GENERAL INTRODUCTION

Monoclonal antibodies (mAbs) have been fully integrated into the treatment paradigms of human disease. This is underlined by an increasing and accelerating number of new therapeutic antibodies being approved every year by the FDA and or EMA, with 10 new approvals in 2017 by the FDA alone [1]. The broad potential of therapeutic antibodies is further emphasized by the breadth of the therapeutic area of the approved antibodies in 2017: 10 approvals in 9 different disease indications, ranging from autoimmune disorders to cancer but also including infectious disease, asthma and hemophilia. Additional differences that stand out are the variety of antibody isotypes employed (IgG1, IgG2 and IgG4) and the diversity in antibody modalities (unconjugated, effector-function adapted, antibody drug conjugate and bispecific). This shows the fit-for-purpose principle of therapeutic antibodies.
Therapeutic antibodies, or immunoglobulins (Ig), are often of the IgG isotype (or derivatives thereof). The structure of antibodies of IgG antibodies can be divided into a Fab region and an Fc region (Figure 1), both contributing to the functional activity. In humans there are 4 IgG isotypes: IgG1, IgG2, IgG3 and IgG4, each containing a distinct Fc region. Functional differences between the isotypes are mainly found in the hinge and CH3 region altering the flexibility, spacing and stability of the molecule [2].

The Fab region is responsible for specific antigen recognition and as such contains a very high level of sequence diversity in order to counteract the enormous repertoire of possible shapes that may be encountered by the immune system. The Fc region provides the bridge between antibody-mediated adaptive immunity (Fab domain) and effector functions of the innate immune system (Fc-mediated effector functions). Therapeutic antibodies targeting CD20 have been approved since 1997, when the chimeric anti-human CD20 monoclonal antibody (mAb) rituximab was approved. This antibody was a chimera of human IgG1 (hIgG1) and the mouse mAb 2B8, genetically fused such that the molecule

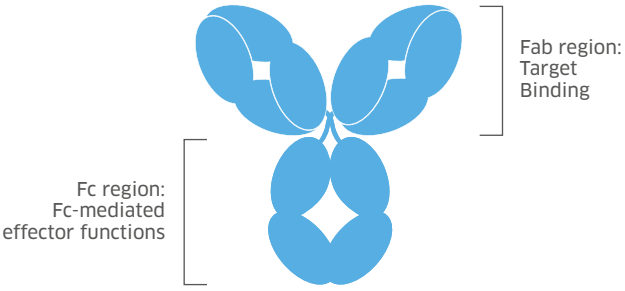

FIGURE 1 **Structure of IgG1.**

contains mouse variable domains and human constant domains. The role of bridging innate effector molecules with the modular response of the adaptive immune system is highly dependent on the isotype of antibody used. A good example of this is the parental clone of rituximab. 2B8 was originally developed as a mouse IgG1 (mIgG1) antibody, and used as such in the product Zevalin® (ibritumomab tiuxetan). In its original mIgG1 antibody format, the antibody is ineffective in Fc-mediated effector functions including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). However when genetically modified to contain a human IgG1 Fc fragment, chimeric 2B8 was capable of inducing CDC and ADCC next to its Fab region-mediated effector functions (Table 1).

Many of the original CD20 targeting antibodies were raised in mice and therefore of a mouse IgG isotype. Mouse IgG exists as four different isotypes: IgG1, IgG2a, IgG2b and IgG3. Based on the interaction with human effectors, mIgG1 is most comparable to hIgG4 and hIgG2, whereas mIgG2a and mIgG2b are more comparable to hIgG1 in the sense that these molecules are able to engage a broader range of Fc receptors and may activate complement. MIgG3 is only able to engage (human) FcγRI [3,4].

In a therapeutic setting (using human effector components), CD20 targeting antibodies of the hIgG1 and mIgG2a isotype can thus bind to C1q and activate the classical complement cascade, resulting in complement dependent cell death (CDC; Figure 2.1). Despite the intrinsic capacity of all (human) IgG1 CD20 targeting antibodies, not all CD20 antibodies induce complement activation efficiently [5]. A feature attributed to the proximity of CD20-antibody complexes that is induced by type I but not type II CD20 antibodies [6,7]. The notion of grouping CD20 antibodies in two types (type I and type II) with distinct characteristics was proposed by Cragg and Glennie [8] and is reviewed in the general discussion.
A second functional difference for type I and type II antibodies lies in their ability to induce significant levels of programmed cell death (PCD; Figure 2.2**).** All CD20 targeting antibodies can induce apoptosis (a form of PCD) when crosslinked via a secondary antibody or Fc Receptors. However, type II antibodies can induce PCD via lysosomal membrane permeabilization (LMP) without the need of additional crosslinking. The ability to recruit effector cells for ADCC or antibody dependent cellular phagocytosis (ADCP; Figure 2.3) is a shared feature of both type I and type II CD20 antibodies.
CD20 down-modulation is a rare mechanism of action of CD20 antibodies and is thought to require an Fc-mediated interaction with FcγRIIb (Figure 2.4) [9]. The role of signaling inhibition/activation has been under-represented in CD20 mechanism of action studies despite it was already described in 1985 by Golay *et al.*, who observed that the CD20 mAb 1F5 induced B cell proliferation whereas CD20 mAb B1 did not (Figure 2.5) [10]. The rarity of this finding is underlined by the fact that after 3 decades of generating CD20 antibodies, mAb 1F5 is still the only antibody reported to induce B cell proliferation.
Antibodies conjugated with drugs or radiolabels use the specificity of the target anti-

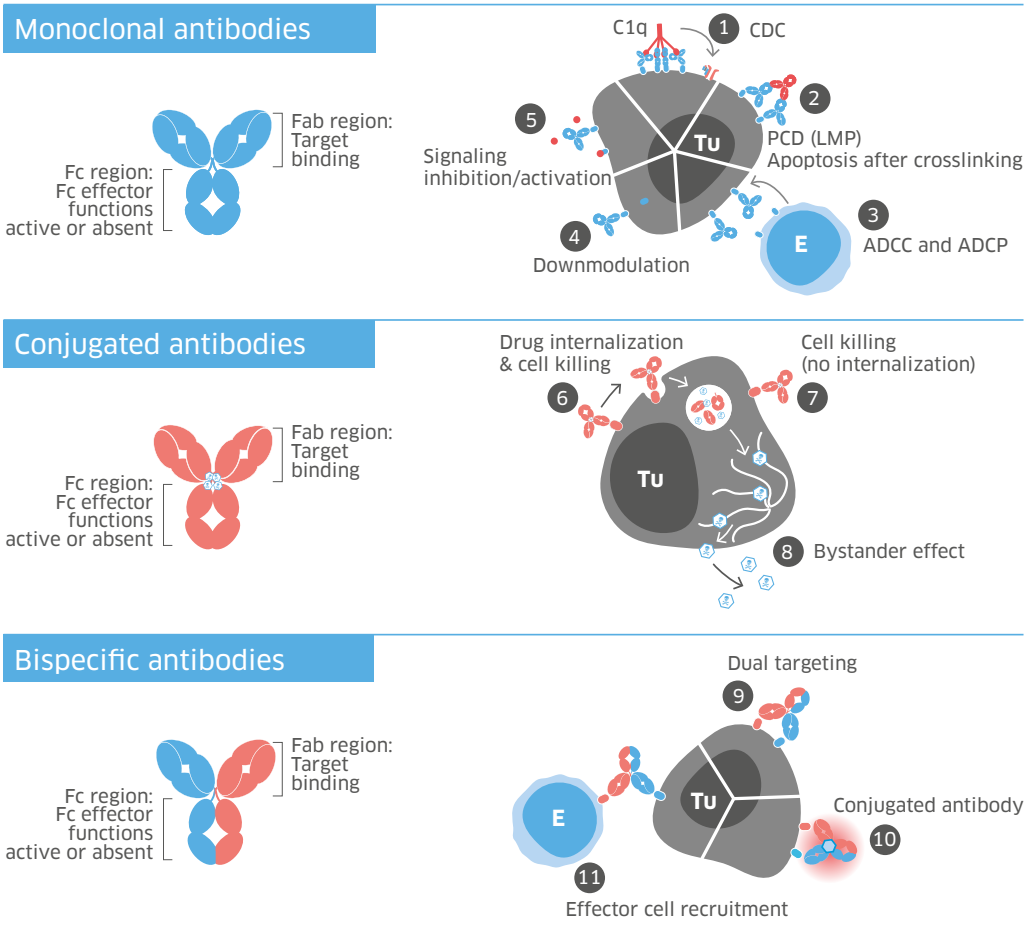

FIGURE 2 **Antibody modalities targeting CD20 and their mechanisms of action.**

body to specifically deliver compounds to CD20+ cells. Most often antibody-drug conjugates (ADC) require internalization and intracellular processing for the induction of cytotoxicity (Figure 2.6), which may be explained by the fact that intracellular release of the toxin (e.g. a tubulin-disruption or DNA-damaging agent) from the antibody is required for the cytotoxic effect to occur. Radiolabeled antibodies in contrast do not require to be internalized (Figure 2.7) as the radiation, typically existing of high-energy beta particles extends beyond the size of a cell. Both radiolabeled antibodies and a subset of ADCs (i.e. those that release membrane-permeable toxin metabolites) have an ability to induce bystander kill in which also neighboring cells are killed whether they express CD20 or not (Figure 2.8).

Bispecific antibodies have the potential to broaden the mechanism of action of CD20-targeting antibodies. Bispecific antibodies may also employ Fc-mediated effector functions or a conjugated toxin to kill tumor cells. Here bispecificity (i.e. the ability to interact with two tumor antigens (or epitopes) instead of one) is used to increase tumor specificity or reduce the chance for tumor escape (Figure 2.9 and Figure 2.10). Bispecific antibodies, which combine targeting arms that binds effector cells (e.g. T cells) with another targeting a tumor antigen (e.g. CD20), represent a particular attractive and promising class of novel therapeutic molecules (Figure 2.11). Extensive experience has been obtained with all the above antibody modalities, mostly in a pre-clinical setting and all are reviewed in chapter 2.

The choice for selecting CD20 as target for immunotherapy and CD20 as target for antibody platform development has various explanations. First, CD20 expression is highly specific for B cells and dependent on development stage as expression starts in pre-B cells and is lost upon differentiation to plasma cells. This indicates that depletion of $CD20^+$ cells does not affect the B lineage stem cell pool allowing for development of new (healthy) B cells. Second, years of development experience with rituximab and other CD20 mAbs has shown that depletion of the $CD20^+$ B cells may induce impressive clinical responses with limited toxicity. Third, a direct correlation between CD20 expression and tumor progression exists for certain malignancies. Fourth, antibody binding to CD20 results in diverse effects on the B cell, dependent on the antibody used. This sparked interest in the role of CD20 in B cell development but also provided tools to study the role of B cells in their interplay between adaptive and innate immunity. Fifth, the availability of a vast amount of tools including immortalized cell lines, *ex vivo* patient material obtained from peripheral blood and the availability of many models for *in vivo* testing allow for a rapid and thorough assessment of antibodies and other reagents. Sixth, the huge amount of public domain data in addition to all the factors mentioned above, make CD20 an attractive target for platform testing as benchmark values and studies with competitor compounds are readily available.

This thesis will follow the evolution of CD20 targeting therapeutics over the past decades. Past experiences with CD20 antibodies have helped us design new, more powerful therapeutic candidates. To understand where the field of CD20 based immunotherapy is coming from and where it is going, Chapter 2 provides an overview of past, present and future CD20 antibody therapeutics, and covers the lessons learned from clinical and pre-clinical targeting of CD20.

Chapter 3 provides a standardized method to quantify and monitor CD20 mAb occupancy on $CD20^+$ cells. This method can be used to follow binding antibody on $CD20^+$ cells over time and as such gives insight in antibody and antigen characteristics. This may provide valuable insights not only in a therapeutic setting but also in the preclinical setting.

The continuous quest to better understand the mechanism of actions used by therapeutic antibodies, and how they interact in the immune network (e.g. how complement activation effects ADCC) led to the discovery of accessory CDC. Here, CDC induced by type I CD20 antibodies is increased through the recruitment of the BCR. This novel mechanism of action is studied and discussed in Chapter 4.

Treatment modalities in lymphoma are shifting from combining of antibodies with chemotherapy, to antibodies in combination with small molecule-targeted therapies. Many of these targeted therapies block intracellular pathways that are shared between leukocyte subsets, and inhibition of these pathways may therefore have an (unwanted) impact on leukocyte effector functions. The effects of the small molecules on the mechanism of action induced by CD20 antibodies (both *in vitro* and *ex vivo*) were studied in Chapter 5.

The road to the design of new and more potent therapeutic antibodies led us to the exploration of the Fab-arm exchange procedure to generate bispecific antibodies targeting both the T cell activation antigen CD3 and CD20 to treat B cell malignancies. Selection of a B cell targeting arm, the optimal Fc backbone and the identification and characterization of a highly potent CD3x-CD20 bispecific antibody are described in Chapter 6. Our studies showed that targeting CD20 was a most effective combination for a CD3 redirection approach. The CD3x-CD20 bsAb molecule described has entered clinical development.

Finally, in c**hapter 7,** key findings and insights are summarized in the general discussion of this thesis. A new view on the function of CD20 in B cell development is provided. The relevance of each chapter is discussed and placed in a context of immunotherapy.

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TABLE 1 Antibodies targeting CD20 with their Fc Isotypes and corresponding effector function noted.

Clone	format	Used in	CDC	PCD (after cross-linking)	ADCC/ ADCP	Down modulation (internaliza-tion)	Proliferation induction Yes (Y)/No (N)
2B8	mIgG1	Zevalin	-	+ (+++)	-	+	N
C2B8	hIgG1	rituximab	++	+ (+++)	++	+	N
1F5	mIgG2				++	+	Y
B1	mIgG2	Bexxar	-	++ (++)	++	-	N
2F2	hIgG1	ofatumumab	+++	- (+++)	++	+	N
7D8	hIgG1		+++	- (+++)	++	+	N
Bly-1	mIgG2		-	++ (+++)	++	-	N
Bly-1[1]	hIgG1	obinutuzumab	-	+++ (+++)	+++[2]	-	N
2H7	mIgG2		++	- (+++)	++	+	N
11B8	hIgG1		-	++ (+++)	++	-	N

1 Clone Bly-1 as used in GA101 contains a modified elbow angle

2 Enhanced ADCC is obtained through glycan engineering (defucosylation of the N-linked glycan)