CD20 as target for immunotherapy
Engelberts, P.J.

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Author: Engelberts, P.J.
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General discussion of the thesis

GENERAL DISCUSSION

CD20-based immunotherapy is contributing to the quality of life for lymphoma patients on a daily basis. In a retrospective study in the United States alone, over the period of 1998 to 2013, it was estimated that rituximab (RTX)-containing lymphoma therapy saved an astonishing 279,704 cumulative life-years [1]. However, despite the groundbreaking contribution of RTX and other CD20 antibody-based therapies to the treatment of B cell malignancies, further room for improvement remains. Not only do numbers of NHL cases continue to increase worldwide, many people also still succumb to the disease. In 1990, the worldwide incidence of NHL was 4.7 (males) and 3.6 (females) per 100,000 with 126,300 males and 94,600 females being diagnosed

with the disease [2]. In 2012, 386,000 new cases of NHL were reported worldwide almost doubling the number of cases in 1990 (220,900) [3]. Next to the newly diagnosed NHL patients, an additional 200,000 people died in 2012, meaning that more than half of the patients suffering from this devastating disease could have benefitted from better therapeutic options.

The aim of the research described in this thesis was to reflect on the current status of CD20 targeting immunotherapy, investigate mechanisms of action and develop next-generation therapeutics building on novel insights and technical innovations. First, I will discuss the CD20 antigen and offer a possible explanation for its function based on a literature review covering CD20 and associated proteins. Second, I will

TABLE 1   CD20 antibodies used in the general discussion.

CD20 compound	Clone	Classification	Epitope*	Reference
rituximab (RTX)	C2B8	Type I	A170-P172	[4]
1F5	1F5	Type I	A170-P172	[4]
B1	B1	Type II	A170-P172	[4]
2H7	2H7	Type I	A170-P172	[4]
Obinutuzumab (OBZ, GA101)	Bly-1	Type II	A171-A176	[5]
FMC7	FMC7	Type I	A170-P172	[6]
ofatumumab (OFA)	2F2	Type I	AGIYAPI**+ T159-N163-N166	[7]
BM-ca	BM-ca	Type I/II	P160	[8]
1.5.3	1.5.3	Type I/II	P172	[9]
7D8	7D8	Type I	AGIYAPI + T159-N163-N166	[7]
11B8	11B8	Type II	AGIYAPI + MESLNFIRAHTPYI	[7]
L27	L27	Type I	A170-P172	[4]
L26	L26	NA	intracellular	[10]

*   Critical amino acids identified.

** AGIYAPI represents a continuous stretch of amino acid and was assumed to encompass the small loop
    (residues 74 through 80).

discuss the impact and implications of the findings reported in this thesis and place it into context of improving patient care with the ultimate goal of saving more lives of people diagnosed with NHL.

DISCUSSION ON THE FUNCTION OF CD20

Although the CD20 antigen is a very well-established target for the immunotherapeutic treatment of B-cell malignancies, still surprisingly little is known about the exact function of CD20. Structural and functional studies have mainly focused on the pharmacodynamic effect of antibodies on the target, and not so much on the physiological role of the antigen. Nevertheless, extensive literature covering CD20 exists and combining the pieces of the puzzle paints a picture of a molecule with diverse functions.

CD20 is a non-glycosylated phosphoprotein that is predicted to span the plasma membrane four times [11] and is a member of the membrane-spanning 4A gene family (MS4A). CD20 consists of four transmembrane regions, two extracellular loops and three cytoplasmic domains. Both the amino- and carboxyl-terminus of the protein are located within the cytoplasm, while only a small part of the protein is exposed extracellularly and is thus accessible for therapeutic antibodies. The extracellular domains consist of a large loop of approximately 44 amino acids (aa) and a small one of 5 aa.[1] It has been suggested that CD20 bears only two immunogenic regions that are recognized by all the different therapeutic CD20-specific monoclonal antibodies (mAbs) [4, 12].

CD20 expression is limited to B cells and therefore CD20 often used as a marker to distinguish B cells from other leukocytes. CD20 has high homology with the β subunit of the high-affinity immunoglobulin (Ig) E receptor (FcϵRI) and HTm4, a protein on lymphoid and myeloid cells with unknown function, all of which are encoded by genes located on chromosome 11 [13, 14]. All these molecules contain a pattern of proline residues within their amino termini, cysteine residues within the extracellular domains and a conserved serine/proline motif in the carboxy-termini, possibly alluding to shared features in downstream signaling. The carboxy-terminus carries an overall negative charge because of the large number of acidic residues located within this region of the molecule [15].

CD20 regulates intracellular free calcium levels
The role of CD20 as a calcium channel or at least a conductor of cytoplasmic calcium regulation is well established. Transfection experiments where CD20 was introduced

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1 The number of amino acids in the small loop has been a subject of debate in the literature in which it ranges from 5 to 9. In this review, a size of 5 aa was chosen because alignment of the MS4A family show consistent hydrophobic amino acids (e.g. Isoleucine) on opposite trans membrane stretches, furthermore the more rigid aa proline is often found at the edge of secondary structures. It should also be noted that the surface exposed area of both loops may be impacted by membrane (e.g. cholesterol) content as discussed below.

into cells that normally do not express CD20, such as the human T cell line Jurkat and the mouse fibroblast cell line 3T3, resulted in upregulation of cytosolic free Ca^{2+} in these cell lines [16, 17]. The transfection of CD20 into 3T3 cells was sufficient to accelerate G1 progression induced by the cytokine IGF-1, clearly demonstrating a role for CD20 in the proliferation [17]. On mantle zone phenotype B cells ($IgM^+IgD^+CD20^{dim}$), binding of mAb 1F5 induced proliferation, whereas in B cells with a germinal center phenotype ($IgM^{dim}IgD^-CD20^{high}$) no proliferation was induced by 1F5 [18]. Differentiating effects after ligation were also obtained with other B cell subsets, as discussed below. Using blood and tonsillar B cells, Clark and Shu demonstrated that ligation of CD20 with 1F5 resulted in proliferation of tonsillar, but not blood-derived B cells [19]. Other studies using tonsillar B cells showed that resting tonsillar B cells transitioned from G_0 to the G_1 phase after binding of mAb 1F5, but these cells required an additional stimulus to proliferate [20], whereas the more activated tonsillar B cells responded to 1F5 stimulation by proliferation [19]. Proliferation was not induced by monovalent antibody fragments, indicating the importance of crosslinking for this activity [21]. Interestingly, mAb B1 did not induce this effect whereas both B1 and 1F5 blocked differentiation of B cells to immunoglobulin-secreting cells. Also antibody 2H7 did not induce B cell proliferation [21]. This excludes that the observations can be linked to the type I/type II classification of CD20 antibodies that groups CD20 in antibodies with distinct functional activity (indicated in Figure 3). This classification will be further discussed in the second section of this review [22]. The distinct effects of CD20 antibodies on cell cycle progression but not on inhibition of B cell activation, imply that CD20 has a dual role in B cells depending on activation or differentiation state, which is further underlined by the differences and heterogeneity found in CD20 phosphorylation between normal B lymphocytes and Burkitt lymphoma cell lines [23, 24] and their response to extracellular calcium [17].

Site-specific CD20 phosphorylation is dependent on kinase.

Studies addressing CD20 function have been clouded by the different results obtained on resting B cells versus activated B cells, resulting from differential phosphorylation of the intracellular c-terminus of CD20.

In resting B cells, the phosphorylation of CD20 is modulated by protein kinase C (PKC) [25], and can be inhibited by the PKC inhibitors palmitoyl carnitine or H-7 [26]. Phosphorylation of CD20 results in an increase in intracellular calcium from intracellular stores (but not from extracellular source) in a phospholipase C (PLC)-γ dependent manner [27] and is down-regulated after ligation of the B-cell receptor (BCR) [25]. This suggests a role for CD20 in B-cell maintenance through steady-state B cell turn over and survival signals which are altered by activation signals such as those provided by the BCR.

In activated B cells, as well as B cell lines and malignant B lymphocytes, CD20 is constitutively phosphorylated. This can be further increased by binding of mAb B1 or other stimuli [24]. This phosphorylation of

CD20 is modulated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II)-kinases in response to calcium levels [28, 29]. CaMK-II phosphorylates CD20 predominantly at Serine 231 and Serine 289, which differs from the site where PKC-mediated CD20 phosphorylation occurs [28].

Both in resting and in activated B cells the role of CD20 is to provide sufficient intracellular free calcium. However this is achieved by different mechanisms. In resting B cells, CD20 phosphorylation leads to the release of calcium from intracellular stores, in activated B cells the free calcium is provided by a CD20-mediated extracellular calcium influx.

CD20 translocation into lipid rafts is required for its function

The influence of cholesterol content on membrane rigidity and its impact on CD20 conformation was first described using mAb FMC7. FMC7 is as an antibody that recognizes a conformational epitope on CD20. Recognition of CD20 by FMC7 is relatively sensitive to the cell membrane's cholesterol content, compared to recognition by other CD20-specific antibodies, which require more severe cholesterol depletion to be affected [6, 30]. Blocking intracellular synthesis of cholesterol by statins as means of stringent cholesterol depletion [31], resulted in reduced CD20 mAb binding and efficacy. The decrease was likely due to masking of epitopes on CD20 rather than to disruption of lipid raft translocation or reduced surface expression. This indicates that CD20's surface exposure is dependent on, and may be regulated by membrane cholesterol content.

Besides influencing the detection of CD20 by antibodies, cholesterol plays a crucial role in lipid raft assembly [32]. Lipid rafts are essential for signaling events in B cell differentiation and response. A domain in the C-terminal, cytoplasmic tail of CD20, close to the lipid bilayer (aa 219-225), was demonstrated to be essential for CD20 translocation into lipid rafts [33], owing to a post-translational palmitoylation of the cysteine residue at position 220 [34, 35]. Palmitoylation is a reversible attachment of a saturated fatty acid to cysteine residues and serves as a lipid anchor for proteins which allows relocalization of the protein in the cell membrane [36].

The activation of G protein-coupled receptors is regulated by reversible palmitoylation and cholesterol levels [37, 38]. Here, cholesterol has a direct effect (by interacting with the palmitoylation), but also an indirect effect by orchestrating the lipid raft domains wherein GPCR-mediated signaling is enhanced.

Like with GPCRs, cholesterol appears to play direct and indirect effects on CD20-mediated signaling. On the one hand CD20 signaling requires its translocation in cholesterol-rich lipid rafts and on the other hand it serves as an anchor for the palmitoylation at Cys220. The reversible phosphorylation and palmitoylation can also provide a switch between activation and inhibition possibly explaining differential effects [39].

There is clear evidence that the BCR and CD20 share intracellular pathways, as ligation of CD20 or BCR induces similar transcription patterns [40]. Furthermore,

activation of the BCR results in its rapid translocation to lipid raft micro-domains in close proximity to CD20 [41] followed by activation-dependent dissociation [42]. CD20 activation requires translocation to lipid rafts, but distinctive CD20 phosphorylation results in different downstream pathways [25,28]. This suggests that in these lipid rafts different signaling molecules are recruited. The association of CD20 and the Src-family was shown to be dependent on the binding of RTX to CD20 and the subsequent translocation to lipid raft domains [43]. The temporary recruitment of Lyn to CD20-rich lipid rafts upon RTX binding was regulated by the transmembrane adaptor protein Cbp/PAG (Cbp; Csk-binding protein/PAG; protein associated with glycosphingolipid-enriched micro domains) after which Lyn disassociated from the raft domains [44,45].

This complex role of CD20 activation that can lead to different effects might be explained by distinct conformations of CD20 tetramers [5,46].

In early B cells, cholesterol content influences the signaling of the pre-BCR [47] whereas in later stages of B cell differentiation FcγRIIb associates with lipid rafts to regulate ligand binding activity [48].
BCR signaling is key for the adaptive immune response, which is also highly dependent on lipid rafts as suggested by the reduction in the IgM BCR-induced calcium flux upon depletion of membrane cholesterol [49]. Because BCR signaling is critical at the different stages of development of a B lymphocyte it is not surprising that many B cell malignancies and other B cell deficiencies are linked to defective BCR signaling. In chronic lymphocytic leukemia (CLL), where

the BCR often recognizes autoantigens [50] or microbial proteins [51,52], BCR crosslinking-induced signaling is distinct from that of normal B cells [53]. In mantle cell lymphoma (MCL), similar to CLL, the BCR signaling cascade is different from that of normal B cells [53]. The role of the BCR in the pathogenesis of MCL has not been fully elucidated yet but there is a bias in IGHV-gene repertoire selection and its immunoglobulin sequence is close to germ line [54]. In Both CLL and MCL, the role of the BCR signaling pathway in disease pathogenesis by was underscored by the efficacy of ibrutinib in the treatment of these diseases. Ibrutinib inhibits the BCR signaling pathway by targeting of the tyrosine kinase BTK. Also developmental defects such as class switch impairment, lead to defects in BCR signaling. This was observed by a decreased calcium flux upon IgM BCR cross linking and dissociation from the activating complex [55].

Proposed dual mechanism of CD20

Taken together, this proposes a dual role for CD20 on B cells. First, on resting B cells, CD20 is required for providing calcium from intracellular stores for steady state B cell turnover. This is through a PKC dependent mechanism, which phosphorylates CD20 at positions S35 and S36 (Figure 1A). On activated B cells, BCR signaling through antigen binding (or alternatively by ligation of the BCR by CD20 antibodies) results in the accumulation of the BCR, CD20, PAG and Src-family members in lipid rafts (Figure 1A).

In lipid raft domains the CD20 molecules are positioned as tetramers, functioning

as a calcium channel that provides the intracellular free Ca^{2+} levels required for BCR signaling. CaMK-II senses the calcium levels and, when a threshold of intracellular calcium is reached, phosphorylates the Serines at position 231 and 289 of CD20. Phosphorylation increases in the depalmitoylation rate of the intracellular cysteine (Cys 220) at the C-terminus of CD20, thereby dissociating CD20 from lipid rafts and stopping calcium influx by rearranging CD20. The BCR signaling complex is then internalized as a negative feedback loop for the activation signal (Figure 1B).
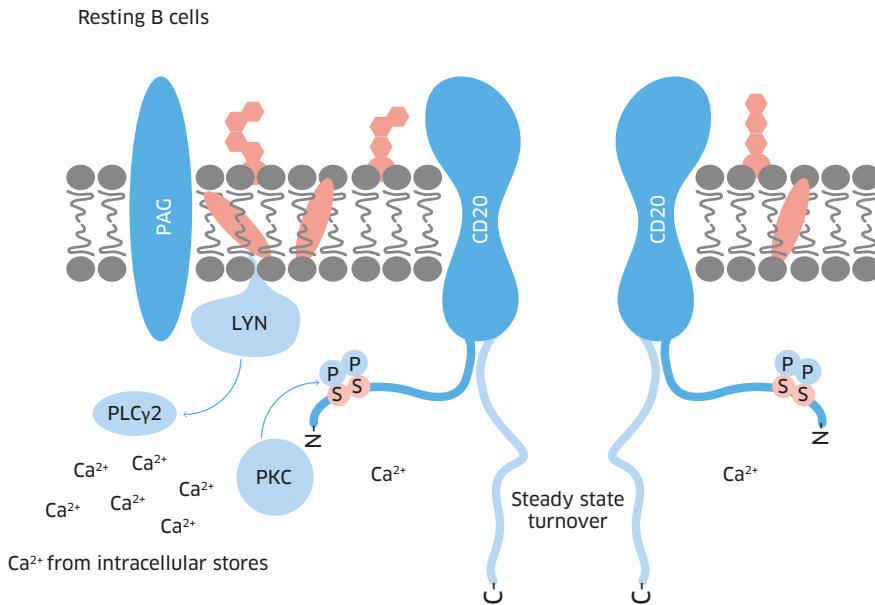

FIGURE 1 **The role of CD20 in resting B cells.**
In resting B cells the lipid raft complex contains CD20 and the signaling molecule LYN and adapter molecule PAG. CD20 phosphorylation by constitutively active PKC leads to proliferation and survival signals of these B cells.

FIGURE 2 **The role of CD20 in activated B cells.**
BCR-mediated activated B cells signal via a lipid raft signaling complex. **1.** Palmitoylation of the C-terminus of CD20 facilitates the relocation of CD20 in BCR-containing lipid rafts, where the ability of CD20 to function as a calcium channel provides intracellular free calcium, required for the BCR signaling cascade. **2.** CaMK-II senses intracellular calcium and phosphorylates CD20, leading to the dissociation of CD20 from BCR-associated lipid raft complexes, upon which the BCR is internalized and CD20 diffuses and no longer functions as calcium channel.
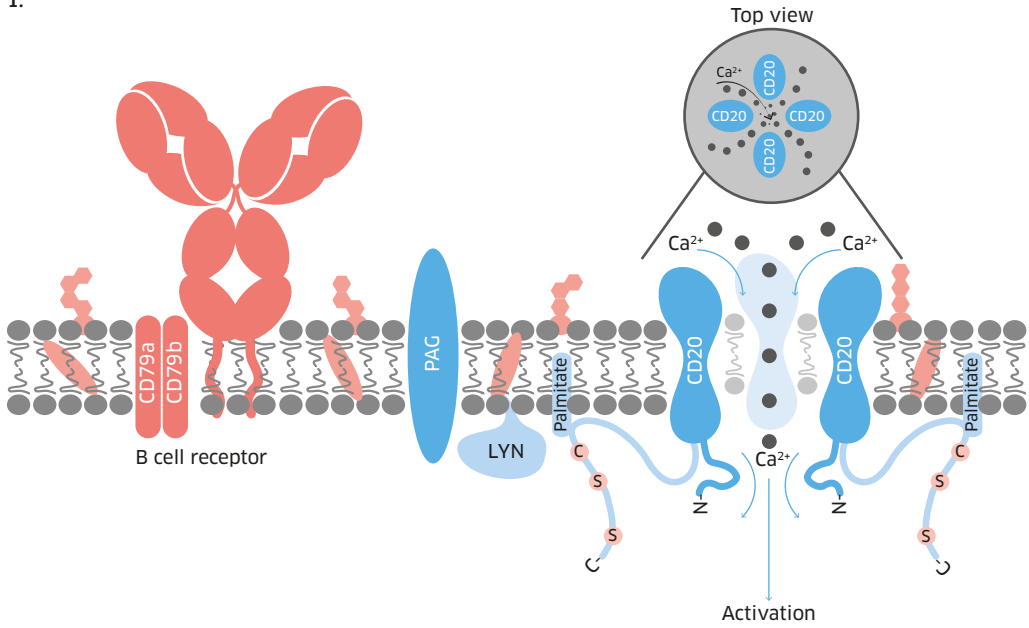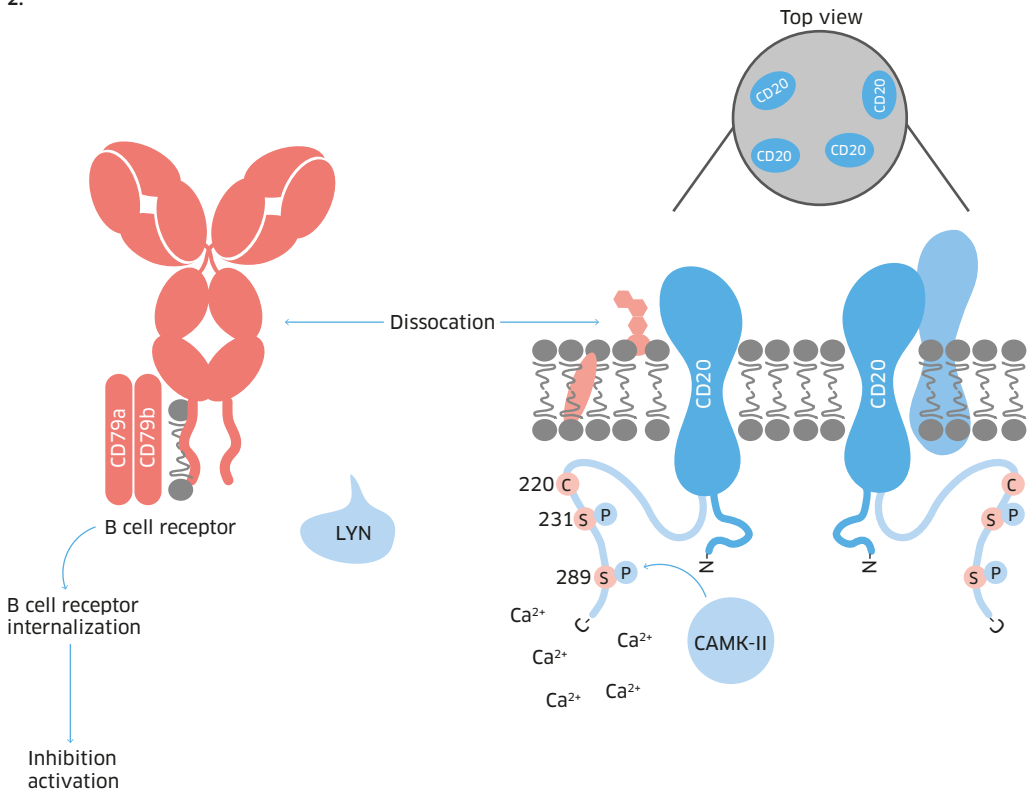
**Current classification of antibodies
targeting CD20**

In Chapter 2 of this thesis, CD20 is reviewed as a target in immunotherapy. In this review the evolution of immunotherapy follows the development of new pre-clinical ideas and compounds to target CD20 and their eventual transition into clinical investigations. The characteristics of the B-cell specific surface marker CD20 have been central to this evolution. On the one hand there continues to be an unmet medical need for more effective CD20-targeting agents and, on the other hand, as clinical experience shows, depletion of CD20+ cells is relatively safe, thereby making CD20 a highly suitable target for development, allowing the validation of novel antibody molecules and antibody-based therapeutic regimens. It is therefore not an exaggeration to state that the development of CD20-targeting agents has been instrumental and of the utmost importance for the therapeutic antibody field as a whole.

The generation of mAbs targeting CD20 is challenging since only a small portion of its 297 aa acids is surface exposed. Still, a variety of monoclonal antibodies (mAbs) targeting CD20 have been generated which largely fall into two groups based on their binding regions (epitopes). Site-directed mutagenesis studies provide a means of delineating binding epitopes of CD20 mAbs in some detail. These studies are performed by using human-to-mouse amino acid substitutions (or vice versa) in CD20 and assessing loss or gain of binding. The use of sequence substitutions from the mouse/human homologue is thought to prevent the occurrence of gross conformational changes providing false-negative results. Using this approach to study the binding region of the original mAbs (many derivatives of e.g. the 2B8 and 2H7 clones are reported), two immunodominant regions in CD20 are identified (Figure 3A). First, there is a region identified by binding of RTX, where antibodies typically require the alanine at position 170 and the proline at position 172 for binding [4]. Antibodies binding to this region include mAbs B1, 2H7, 1F5 and L27 but also Bly-1 (GA101; obinutuzumab (OBZ)) [5, 12] and 1.5.3 [9]. Second, there is the region identified by binding of ofatumumab (OFA) which requires small loop amino acids together with aa residues T159, N163 and N166 in the large loop [7]. The group of antibodies binding to this region includes 7D8 and 2C6, but also OUMB6, OUMB3 [56], BM-ca [8], UMABs [57] and 3B9-10 (i.e. the CD20 targeting arm of the Regeneron CD3xCD20 bispecific antibody (REGN1979)) [58].

Several approaches have been used to distinguish the various properties of the CD20-targeting antibodies. Crystal structure analysis of selected CD20-targeting antibodies, in complex with CD20-derived peptides, shows that some antibodies (C2B8/RTX and C2H7/ocrelizumab (OCRE)) bind to identical epitopes [59,60]. Similarly, crystallography studies comparing RTX with OBZ show that the latter recognizes a slightly different, but overlapping, epitope, resulting in a different binding orientation [5]. Indeed, the impact of binding characteristics on the antibodies' mechanism of
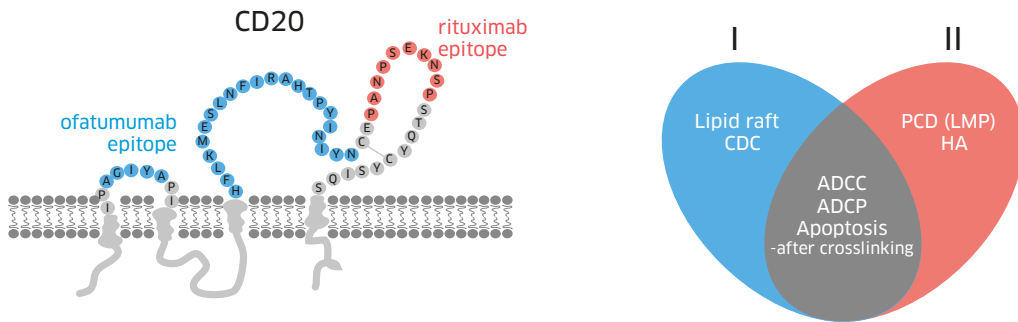
AGIYA - FIRATPYINIYN	PANPSEKNSPS	Clone	Type I	Type I/II	Type II
	▬ (red)	RTX, 2H7, 1F5, FMC7	▬ (blue)		
▬ (blue)		2F2, 7D8, 2C6	▬ (blue)		
	▬ (red)	B1			▬ (red)
	▬ (red)	Bly-1			▬ (red)
▬ (blue)		11B8			▬ (red)
▬ (blue)		BM-Ca		▬ (gray)	
	▬ (red)	1.5.3		▬ (gray)	
▬ (blue)		OUMB6	▬ (blue)		
▬ (blue)		OUMB3	▬ (blue)		
▬ (blue)		REGN1979	▬ (blue)		

FIGURE 3   The two ways of classification of CD20-directed antibodies.
Currently classification of CD20 antibodies is done in two ways, either based on epitope or based on effector functions. On the left, classification based on binding to one of the immunodominant regions is shown. The RTX region contains the AxP motif and surrounding amino acids. The OFA region contains amino acids from the small loop (AGIYA) and large loop (T159-N163-N166) other than that important for binding of rituximab. On the right side classification based on effector mechanisms is shown, antibodies are classed as either type I or type II.

action is brought home most strongly when considering OBZ development. OBZ's mouse antibody parent Bly1 is a type II antibody (Figure 3B). After humanization, the resulting antibody however obtained type I characteristics, which could be restored to type II activity in OBZ by modifying the elbow region [61]. A crystal structure of OFA in complex with CD20 is not available, since peptides recapitulating the OFA epitope could not be generated due to its complex quaternary structure encompassing amino acid residues in both the small and large extracellular loop. However, the crystal structure of the Fab fragment of OFA was believed to give some insight in its binding properties, as hydrophobic residues were identified which were suggested to allow

binding close to the membrane [62]. In the absence of crystallography studies and with mutagenesis studies not being differentiating, new techniques such as cryo-electron microscopy (cryo-EM) are envisioned to shed more light on the structure of the intact CD20 molecule. This also is expected to provide further insight into how mechanisms of action of CD20 antibodies can be almost unrelated to their binding region. Another way of classifying CD20 targeting antibodies is by functional activity based on their mechanism of action. Originally Polyak and Deans classified CD20 antibodies into four groups based on binding to aa residues A170 and P172 (a.k.a. the AxP motif), homotypic aggregation (HA; i.e. the ability of CD20 mAbs to induce cell-cell clustering) and CD20 translocation to lipid rafts [4]. Later this classification was updated by Cragg and Glennie [22]; based on antibodies 1F5, RTX and B1, they proposed the type I and type II classification. Type I anti-CD20 antibodies redistribute CD20 into lipid rafts, are efficient in complement-dependent cytotoxicity (CDC) and type II anti-CD20 antibodies do not redistribute CD20 into lipid rafts, are unable to elicit CDC, yet are able to induce programmed cell death (PCD) through an Fc-independent mechanism (Figure 3B).
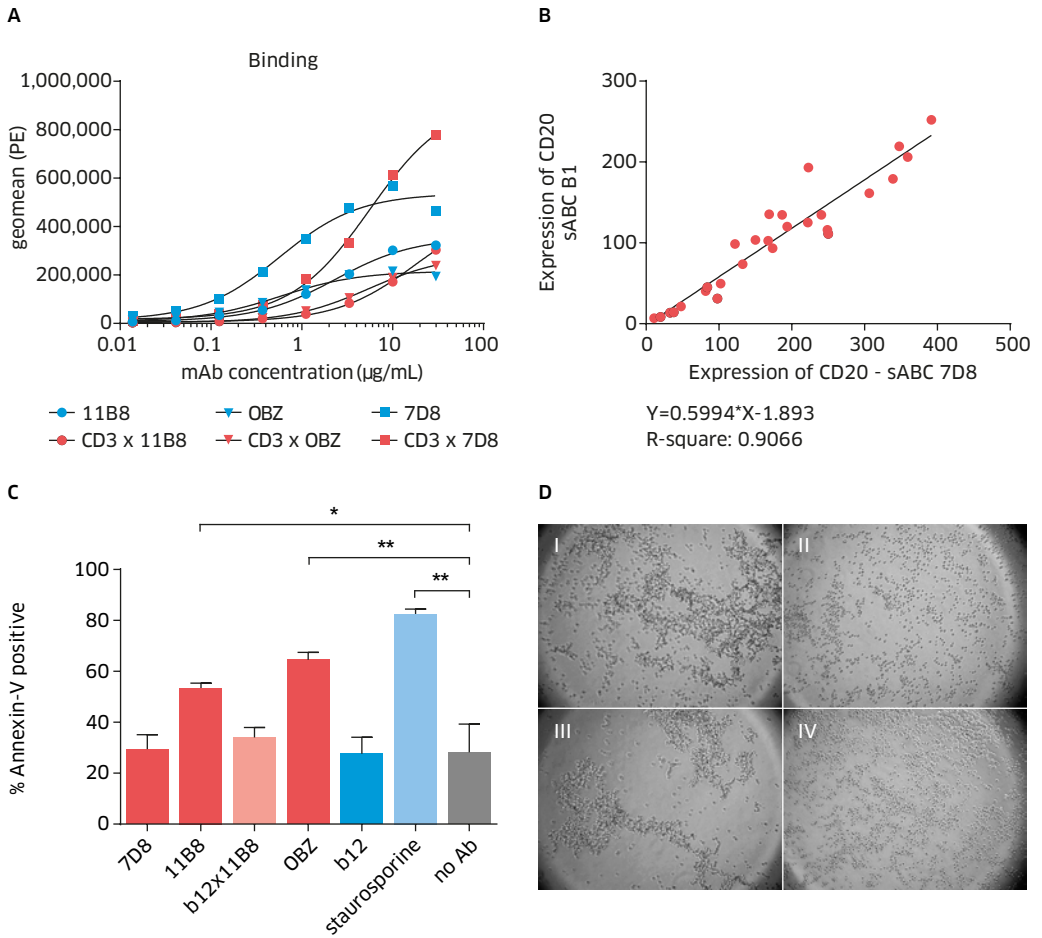
Despite the clear distinction in several key functional activities as a result of CD20 mAb binding to cells, controversy still exists. First, there is some contradictory information on the reported induction of apoptosis by CD20 mAbs as previously pointed out by Deans *et al.* [63]. Second, most reported apoptosis induction required additional crosslinking and was based on

Annexin-V/PI staining, which is a generic hall mark for programmed cell death (PCD) of which apoptosis is a subtype [64]. A key parameter for apoptosis is the activation of caspase-3 of which in most experiments, evidence is lacking. Additionally Alduaij *et al.* showed that in fact type II CD20 mAb-induced cell death represents a different subclass of PCD: lysosome-dependent cell death (LCD; lysosome swelling followed by membrane permeabilization resulting in a caspase independent, cathepsin induced cell death) [65]. Third, two separate reported entities, lipid raft localization and CDC appear to be correlated [66] as target-enriched regions facilitate antibody clustering and efficient C1q docking. Despite these challenges in classifying CD20 antibodies, the current consensus is that type I antibodies, including RTX, OFA but also 2H7, 1F5, L27, 7D8, 2C6, OUMB6, OUMB3 and 3B9-10, translocate CD20 molecules into lipid raft domains, induce CDC, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP). Whereas type II antibodies, including B1, Bly-1 and 11B8, do not translocate CD20 molecules into lipid rafts, induce lysosomal-dependent cell death, ADCC and ADCP. Upon crosslinking all CD20 directed antibodies induced a form of PCD that was dependent on caspase-3 and thus represents apoptosis. The ability to induce homotypic aggregation (HA) and the observation that binding occurs at approximately half the maximal occupancy represent other characteristics in which type II antibodies can be distinguished from type I antibodies. HA occurs via Fab-mediated cellular crosslinking accompanied by intracellular actin polymerization [65].

The lower maximal antibody occupancy for type II as compared with type I antibodies, revers to a phenomenon where about twice the number of the type I antibodies can bind to a single B cell as compared to type II antibodies, suggested to be a consequence of a different recognition of the epitope resulting in a distinct angle of binding [12]. Using a bispecific antibody (bsAb) format, containing a CD20 and an irrelevant binding arm (i.e. functionally monovalent bsAb), we demonstrated that functionally monovalent type I CD20 antibodies have more antibody-binding sites per cell (ABC) than the bivalent parental mAb. In contrast, functionally monovalent type II bsAb showed similar binding compared to the bivalent parent mAb. This indicates that type II CD20 mAbs bind monovalently on the surface of CD20+ cells. This conclusion is supported by the EC_{50}, which differs for monovalent and bivalent type I CD20 mAbs but is similar for type II antibodies in bsAb and mAb format ((Figure 4A+B, unpublished data). Presumably, monovalent binding and the distinct binding angle lead to steric hindrance, thereby effectively reducing the maximal number of available antibody binding sites for CD20 mAbs on the cell surface. Both the HA and PCD activity of type II CD20 are shown to be dependent on their ability to bind bivalently (Figure 4C+D, unpublished data), suggesting that a quality of these antibodies is that they crosslink CD20 molecules between cells.

Whilst this classification of CD20 antibodies is a very useful means of distinguishing between the intrinsic mechanisms of CD20 antibodies, it is becoming difficult to maintain. BM-ca [67] and 1.5.3 [9] were the first

antibodies to deviate of the classification and were termed type I/II based on their *in vitro* ability to recruit all mechanisms attributed to both type I CD20 antibodies and type II CD20 antibodies. Later, antibody engineering, such as Fc engineering [68], was employed to increase one or multiple mechanism of action. Although these engineered antibodies were still classifiable as type I or type II, the capacity to induce cell death *in vitro* via the improved mechanism of action was enhanced or selectively decreased [69]. One undermining factor is that over-interpretation might play a role when singling out effector functions. One example is the interplay between complement activation and complement dependent cell-mediated cytotoxicity (CDCC; complement-enhanced ADCC) and complement-dependent cell-mediated phagocytosis (CDCP; complement-enhanced ADCP) [69]. Furthermore, despite the fact that PCD is considered a Fab-mediated effector mechanism, alterations in the Fc-fragment may affect the magnitude of PCD [70]. Moreover, evidence exists that some of the Fab-mediated effector functions might be overestimated as a result of assay-induced phosphatidylserine (PS) externalization [71] or the use of a non-specific antibody [72]. As new CD20-targeting antibody formats such as bispecific antibodies (e.g. CD3 BsAbs discussed in chapter 6) are strictly monovalent for CD20, the need to classify them as type I or type II is less compelling and could be abandoned or restricted to original clones.

FIGURE 4 Type II CD20 characteristics can be divided into monovalent and bivalent interactions.
A. mAb and bsAb of type II antibodies OBZ and B1 display similar maximum antibody occupancy. In contrast, the type I CD20 antibody 7D8 displays a maximum antibody occupancy that is higher for the bsAb compared to the mAb. B. the lower maximum antibody occupancy of type II CD20 antibody B1, compared to type I CD20 antibody 7D8, is consistent in approximately 30 different B cell lines tested (**B**). Differences found in PCD (**C**) and HA (**D**) of mAb versus bsAb type II CD20 antibodies suggest that these mechanisms of action result from crosslinking between cells. I. 11B8 mAb; II. ctrlx11B8 bsAb III. OBZ mAb; IV. ctrlxOBZ bsAb. Statistical analysis was performed with ANOVA,* =p<0.05, **=p<0.01.

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Surface CD20 expression can serve diagnostic, predictive and prognostic purposes.

CD20 expression is limited to B cells and as such is used to phenotypically differentiate B cells from other immune and non-immune cells. However the level of CD20 expression is associated with the differentiation state of the B cell. This is reflected in the malignant counterpart of the differentiation state. The leukemic malignancies ALL and CLL originate from early stages of B cell development and therefore have considerably lower CD20 expression compared to normal B-cells from healthy donors [73]. Also, monitoring the levels of CD20 (both as expressed in percentages positive cells, as well as the number of CD20 mAb binding sites expressed per cell (ABC)) gives valuable insights in phenotyping malignancies and levels are predictive for outcome. In Chapter 3, we explore CD20 antigen expression as a tool to monitor the efficacy of CD20 -directed antibody treatment. Antibody characteristics such a target occupancy and off-rate can be monitored in the blood of patients, together with antigen internalization (by detecting the ratio between free and available antigens). All these characteristics are considered to have an impact on antibody-induced effector functions. Besides monitoring CD20-bound antibodies, the levels of CD20 antigen occupied by antibody could also be a predictive tool to study target surface expression levels prior to assigning a therapeutic regimen, as *in vitro* studies have shown that antigen expression levels can be a rate-limiting step for specific antibody-induced mechanisms of action.

CD20-directed therapy can influence CD20 antigen expression, both *in vitro* and *in vivo*. Continuous exposure of cell lines to RTX resulted in reduced CD20 expression [74,75]. Besides altering expression, factors influencing the detection of CD20 also exist. For instance, IL-4 induces a conformational change of CD20 that influences detection of CD20 by antibody L27 in contrast to detection by seven other CD20 antibodies tested [76]. We showed that depletion of cholesterol hampers the detection of CD20 by CD20-targeting therapeutic antibodies [31]. Furthermore, therapy-driven reduction of CD20 expression has been observed. First, downregulation of CD20 through selection of low CD20-expressing B cell lines or the induction of transcription variants has been described following CD20-directed therapy [77-82]. Second, antigen exchange from target cells to effector cells has been shown to occur via a process called trogocytosis (or shaving), impacting the expression of CD20 on both target cells as well as effector cells [83-86].

Third, certain drugs studied in combination with CD20 mAbs have been demonstrated to affect CD20 expression. For instance, lenalidomide reduces CD20 surface expression through increased internalization [87], whereas histone deacetylase (HDAC) inhibitors upregulate CD20 expression [88]. Monitoring B cell counts and CD20 expression before, during and after therapy clearly serves multiple purposes. First, it might serve diagnostic purposes, as the increased presence of B cells in the circulation or in lymph nodes might indicate the presence of leukemia or lymphoma, and, in addition, the level of CD20 expression might be an indication for the subclass of malignancy.

Second, it could provide a tool to select a therapeutic agent. Pre-clinical data has shown that OFA also displays efficacy for B cell malignancies with low copy numbers of CD20, where RTX is less effective. Third, it could reveal a relapse of the disease by B cells devoid of CD20 expression. While this is rare, it exists and these patients need an adapted therapeutic strategy [89]. Fourth, detection of CD20 also serves the purpose of monitoring therapeutic efficacy as the aim of immunotherapy is depletion of B cells. A lack of B cells in circulation (or lymph nodes) is therefore an indication for efficacy, but also remaining debris of killed B cells can serve as a prognostic marker [90]. It should be noted that all known CD20 antibodies (against extracellular epitopes) compete with each other since CD20 is a relatively small antigen. Monitoring of CD20 expression should therefore always be interpreted with care and accompanied by a pharmacokinetic analysis.

New insights in antibody therapy

Despite the fact that immunoglobulin-based therapy has been around for decades there is still ongoing debate on which mechanism of action is contributing to the therapeutic efficacy. Research aimed at elucidating the role of complement to the *in vivo* efficacy of anti-CD20 targeting antibodies led us to unexpected findings, which we discuss in chapter 4. We demonstrated that the recruitment of the BCR to the lipid rafts of CD20 resulted in an accessory CDC aiding the CDC induced by type I CD20 antibodies. This unexpected involvement of the BCR in eliciting CDC was recently suggested by Evers *et al.*, in response to our publication, to also be true for other, yet to be identified

B cell surface molecules. Considering the diversity in ways in which complement can contribute to immunity and especially B cell development this is not surprising.

Hexamerization of antibodies through Fc:Fc interactions allowing efficient C1q docking and complement activation was demonstrated to be a first step in IgG-mediated complement activation [91,92]. Indeed, an extraordinary ability of CD20 to oligomerize on cell surfaces and thereby facilitating hexamerization for OFA-type antibodies, has previously been suggested to be part of the mechanism underlying the high potency of OFA to induce CDC [93]. Our data suggest that surface-expressed BCR may provide an additional complement factor C1 binding and activation site [94]. Presumably, clustering of CD20 molecules induced by type I CD20 antibodies may lead to the formation of BCR multimers that allow an avidity interaction with C1 leading to complement activation. Interestingly, molecules involved in complement regulation are also found in lipid rafts, which suggests a functional relation. For CD55 and CD59 (i.e. GPI-anchored complement defense molecules), which inhibit the terminal pathway of complement activation, this functional relationship would be protection from the accessory CDC. However for complement receptor (CR)2 (CD21) and CR1 (CD35), the role is more complex. CD21 is part of the BCR co-receptor complex, together with CD19 and CD81 [95]. Signaling via BCR and CD21/CD19 was shown to promote B cell survival after a primary immune response [96]. Thus, antigen linked to complement component C3b resulted in more surviving B cells compared to B cells exposed to

antigen alone. Another role of the complement receptor is facilitating antigen presentation in germinal centers (GCs). In a study by Zhang *et al.* it was demonstrated that antigen administered together with antibody resulted in better penetration and presentation of the antigen in the GC than administration of antigen alone. This retention was dependent on CR1/2 (CD35 and CD21) expressed on B cells [97,98]. Interestingly, CD35 clustered with the BCR and B cell differentiation could be inhibited by the addition of aggregated complement factor C3 [99]. Summarized, this data suggests that complement factor C3 halts B cell differentiation in order for B cells and antigen to reach GC, where antigen presentation results in an immune response. Inhibition of the complement cascade by complement defense molecules, to prevent complement-mediated cell death, occurs at the level of activation of complement factors C3/C5 (CD55) or C9 (CD59). This still allows sufficient formation of the ligands for CR1 and CR2. CD20 is a key component in lipid rafts for BCR-mediated signaling in activation. However, this work shows that at least CD35 also plays a role in BCR activation. This could be to eradicate B-cells expressing auto-reactive, complement-activating BCRs. Also, this suggests that complement activation by type I anti-CD20 antibodies could attribute to therapeutic efficacy beyond cell lysis.

Combination strategies in an evolving therapeutic landscape

Small molecules have an important role in the therapeutic arsenal against B cell malignancies. Molecules such as ibrutinib and idelalisib are approved for monotherapy, as well as combination therapy in CLL and dasatinib has been approved for Philadelphia chromosome-positive ALL. In chapter 5 we demonstrated that these small molecules may have multiple on-target and off-target activities, some of which may counteract the mechanisms of action of biologics that are used in combination. For instance, phagocytosis induced by RTX was inhibited by the addition of ibrutinib in a concentration-dependent manner but could be reverted by halting the ibrutinib exposure. This suggests high turnover of the inhibiting effect, but also demonstrates off-target binding, because Bruton's tyrosine kinase (BTK) is not involved in the induction of phagocytosis. Furthermore, it has been demonstrated that ibrutinib antagonizes natural killer (NK) mediated cell kill and IFN-γ production induced by RTX (*in vitro* and *in vivo*) [100].

One of the off-target effects of ibrutinib is a direct inhibition of the interleukin-2-inducible T-cell kinase (ITK) (a member of TEC-kinase family to which BTK also belongs) [101]. In a study monitoring CLL patients on ibrutinib treatment demonstrated that T cell inhibition indeed was a direct effect of ibrutinib. Skewing of T cell responses toward type 1 T helper cell (Th1) responses, suppressing regulatory T cells (Tregs) and reducing PD-1 and PD-L1 expression, in addition, however, represented long-term effects of ibrutinib [102]. This observation is supported by the finding that continuous ibrutinib exposure decreases the expression of inhibitory molecules and potentiates CAR-T cells *in vitro* and *in vivo* [103].

The potential of combining idelalisib with RTX was demonstrated in a phase III study where the investigators compared the combination to RTX only. This study needed to be stopped prematurely due to the significant benefit of the combination over RTX [104]. In a follow-up study, similar results were obtained with OFA and idelalisib [105]. Contrary to these findings, a phase I study where idelalisib plus RTX was compared to idelalisib plus bendamustin or idelalisib plus bendamustin plus RTX, the ORR (respectively 75%, 88%, 79%) seemed to favor the combination without RTX [106]. This could at least partially be explained by the fact that similar to ibrutinib, idelalisib also inhibited the mechanism of action of CD20-directed antibodies, albeit less pronounced [107]. Besides interrupting the BCR signaling cascade, inhibition of the PI3K pathway also affects T cell functions. In mouse studies inactivation of PI3Kδ resulted in altered balance of CD4$^+$/CD8$^+$ cells upon induction of an anti-tumor response [108]. Next to altering the balance of T cells, the PI3K inhibition also results in more active CD8$^+$ T cells [108, 109].

Also for dasatinib, a small molecule approved for the treatment of ALL, off-target effects inhibit signaling in T cells. Dasatinib was approved for its inhibiting effect on the BCR-ABL protein that is derived from the Philadelphia chromosome due to gene translocation. However, a daily dose of dasatinib resulted in a reduction in the number of Tregs (CD4$^+$CD25$^+$CD127^{low}), as well as an increase in the number of differentiated NK cells (CD3$^-$CD57$^+$/CD3$^-$CD56$^+$) [110].

Although it has been demonstrated (at least for ibrutinib) that timing of the administration of either of the drugs used in combination is important [111], this also suggests that potentially better drug combinations than naked CD20 antibodies with these small-molecules could be developed. Similar to the benefit of adding BTK inhibitors to treatments with CAR-T cells, benefit of adding the small molecules ibrutinib or idelalisib to CD3 bsAbs can be envisioned. First, also for the CD3 bsAbs, targeting the PD-1/PD-L1 axis potentiates the efficacy [112, 113]. Second, by increasing the number of CD4$^+$ and CD8$^+$ T cells [114] and activation of the cytotoxic potentials of CD8$^+$ T cells via production of IFN-γ and IL-2, the small molecules and CD3 bsAbs might act synergistically in T cell activation and eradication of neoplastic B cells [115-117].

Future direction in CD20 directed immunotherapy

New therapies are entering clinical investigations with seven newly registered clinical trials focusing on CD3xCD20 bispecific antibody molecules and 15 newly registered trials of CAR-T cells targeting CD20 (www.clinicaltrials.gov, may 2018). Both treatment modalities redirect T cells to kill the target-expressing cells, in this case CD20-expressing cells. Although both the CAR-T cell and the CD3 bsAb approach make use of the cytotoxic potential of T cells their approach is quite different. The *ex vivo* induced expression of a scFv fragment on (autologous) T-cells requires different selection criteria compared to a generic T-cell binding bispecific antibody molecule.

In Chapter 6 we describe the process of evaluating tumor targeting arms in a bsAb to identify the most potent compound that is able to eradicated tumor antigen expressing cells. We found this process to be empirical as no characteristic could be singled out to identify a potent compound. However, in all the efficacy screenings we employed, however, always one combination stood out: DuoBody-CD3xCD20. In chapter 6 we further investigate the *in vivo* efficacy of the novel CD20-targeting bsAb, together with its mechanisms of action.

The CD3 bsAbs targeting CD20 are initially investigated in relapsing or refractory lymphoma patients. A challenging patient pool where first clinical investigation studies are already showing promise but follow-up phase 3 studies are needed to confirm these early response and show the depth of the clinical responses. The potency of the new CD3xCD20 bsAb compounds is expected to be very high as can be distilled from the *in vitro* potency. Another reflection of the expected potency is the difference in amount of compound administrated. The REGN1979 CD3xCD20 bispecific molecule has been tested in a phase 1 clinical study at flat doses ranging from 0.03 – 3.0 mg compared to the standard dosing of RTX in lymphoma (375 mg/kg every cycle). Although phase 1 studies are designed to study safety of the tested compounds nevertheless clinical responses have been observed in at least some of the patients in this phase 1 study indicating efficacy of this compound already at low dose [118]. The final dose of DuoBody-CD3xCD20 needs to be determined in a dose escalation

study and the current study mainly focuses on safety of the drug.

Also, the depth of the B cell-depleting capacity of DuoBody-CD3xCD20, as observed in cynomolgus monkeys, was intriguing as potent depletion was observed in lymph nodes as well as bone marrow, which represent anatomical sites implicated in ineffective therapy due to the providing of protective niches for tumor cells. The forced cross linking of tumor cells and T cells by the bsAb molecule may lead to the initiation of an adaptive memory response when combined with anti-CTLA as was observed by an increase in CD4+ memory T cell expansion [119]. In mouse studies, treatment with anti-CD20 resulted in increased numbers of Th1 cells and decreased numbers of Treg cells compared to treatment with an irrelevant targeting antibody [120]. Adoptive transfer of these CD4+ T cells into naive mice resulted in protection to a subsequent challenge with CD20+ tumor cells.

B cell-depleting agents might hamper the humoral immune response by eradication of B cells, which might serve as antigen presenting cells. Hassan *et al.* demonstrated that in response to LMB-1, an immunotoxin targeting Lewis-Y tumor antigen, all patients developed neutralizing antibodies to the immunotoxin by day 21 despite absence of peripheral lymphocytes as a result of RTX treatment [121]. Even in the presence of RTX, which depletes all peripheral B cells, an immune response against lymphoma was described [122]. Hilchey *et al.* described, in a small study, that 4 out of 5 patients with follicular lymphoma (FL) on RTX monotherapy displayed an increase in

FL-idiotype specific T cells. This interesting observation was followed by several phase I/II clinical studies where RTX therapy was combined with immunogens, such as KLH conjugated anti-idiotype, neoantigens or CpG. So far no updates for these clinical studies are available while we await their results with interest. Another approach was to vaccinate with a plasmid encoding murine CD20. The goal of this study was to raise a long lasting response against CD20. The rationale behind this approach was tested in mice where immunization with a 12-mer peptide derived from CD20 (that was recognized by RTX) and resulted in an immune response to CD20. No development has been reported despite being completed as of 2007.

The role of complement in antigen trafficking to the germinal centers and B cell development was already mentioned in the discussion of chapter 4. However, it was recently shown that complement, specifically C1q, plays a crucial role in antigen cross-presentation and induction of CD8+ T cell responses. FcγRs interestingly were found to be irrelevant for cross-presentation *in vivo*, but not *in vitro*. [123]. As type I CD20 antibodies potently bind C1q on cells [93], this might result in efficient transfer of lysed cell fragments to dendritic cells (DCs) besides the role in clearance of pathogens [92].
Activation of the immune system via a vaccine or activation of the complement cascade might be a way to induce a long-lasting immune responses against the tumor.

To summarize, CD20 plays a crucial role in the development of B cells by partnering with the BCR. During development of the adaptive immune response continuous antigen exposure together with genetic vulnerability associated with gene rearrangements, somatic hyper-mutation and class-switching can lead to the development of lymphomas. The mostly parallel surface expression of CD20 and the BCR during B cell ontogeny, together with the absence of CD20 expression on other cell types makes CD20 an ideal target for immunotherapy of B cell tumors. This is underlined by the clinical activity of RTX, OFA and OBZ.

Clinical and preclinical investigations of antibody molecules or antibody-derivatives are giving us new insights in the requirements for effective immunotherapy. However, personalized medicine will need to play a more prominent role as it is clear that lymphoma is not a uniform disease. Because although lymphomas ironically originate from clonal cell expansion, it is an extremely heterogeneous disease. Even the subclasses in non-Hodgkin's lymphomas such as FL and DLBCL represent relatively coarse designations and contain a multitude of subtypes. Prediction of the response, location of the disease and selection of combination partners are expected to increase the therapeutic responsiveness. Monoclonal antibodies targeting CD20 have been the gold standard to treat lymphoma, but bispecific antibodies and transduced effector cells (e.g. CAR-T cells) are producing stunning data and I anticipate that these will develop into novel game-changing therapeutic agents. A decade of CD20 antibody research led to the development of CD3xCD20 bispecific molecules with great potential, a view shared by many consider-

ing the large number of CD3xCD20 bispecific molecules currently in clinical development. The efficient penetration of bispecific molecules into secondary lymphoid organs, conditional activation of T cells only in the presence of the target and the low dose required to reach a Minimum Anticipated Biological Effect Level (MABEL) are key aspect that in my view will make CD3 bispecifics in general, and DuoBody-CD3xCD20 specifically, write a next chapter in the evolution of CD20 antibody therapeutics.

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