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Review Article

Mechanisms of action of CD20 antibodies

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Abstract: Therapeutic monoclonal antibodies (mAbs) that target the CD20 antigen on B cells are successfully used in the clinic for the depletion of B cells to treat various forms of cancer and autoimmune diseases. The first CD20 mAb, approved by the FDA in 1998, was rituximab (RTX) and since then it has been widely used to treat more than one million patients thus far. The success of RTX has led to a general interest in the mechanism of action of CD20 mAbs. CD20 mAbs can induce tumor killing via various mechanisms, such as direct induction of apoptosis, antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent lysis (CDC). Although we now understand these mechanisms better, it is still unclear which of these mechanisms is the most important for *in vivo* RTX action. Not every patient respond to RTX treatment and eventually the overwhelming majority will experience a relapse. Therefore, there is an urgent need to improve the efficacy of CD20 mAbs. This review aims to summarize our current understanding on the mechanism of action of CD20 mAbs.

Keywords: Antibodies, CD20, effector mechanisms, Fc receptors, complement, complement receptors, apoptosis

CD20 and CD20 antibodies

CD20 is a general B cell marker that is expressed during B cell differentiation from the pro-B cell phase until the plasma cell stadium. The physiological ligand and exact biological function of CD20 is currently unknown. *In vitro* studies propose a role for CD20 as a store operated Ca^{++} channel [1]. Initial analysis of CD20 deficient mice showed no immune-deficient phenotype [2, 3], however, more recently CD20 deficient mice and humans were found to exhibit decreased T-independent immune responses [4]. Largely fuelled by the interest in CD20 as a tumor target, research on CD20 biology has intensified in the recent years. The current knowledge on CD20 biology is summarized in an excellent review by Cragg et al [5].

The chimeric mAb rituximab (RTX, Roche / Genentech) was the first FDA-approved CD20 mAb. It is approved to treat CD20 positive B cell malignancies; e.g. non-Hodgkin lymphoma and chronic lymphocytic leukemia (CLL), and for some autoimmune diseases, including rheumatoid arthritis. RTX is also being used in several other autoimmune diseases, such as systemic lupus erythematosus (SLE) or multiple sclerosis [6]. In oncology indications, RTX is used in combination with chemotherapy [7]. The fully human CD20 mAb ofatumumab (OFA, Genmab / GSK) was approved by the FDA in 2009 for the treatment of CLL patients resistant for both alemtuzumab and fludarabine [8]. Next to the two unconjugated CD20 mAbs there are two radio-conjugated CD20 mAbs on the market used as part of radio-immunotherapy. Ibritumomab tiuxetan (Zevalin) is a CD20 mAb coupled with the radioactive isotope yttrium-90 or indium-111. I-131 Tositumumab (Bexxar) is a iodine-131-labeled CD20 mAb, based on the mouse CD20 mAb B1 and is used to treat follicular lymphoma (FL) patients [9, 10]. This review will focus on the mechanisms of action of unconjugated CD20 mAbs.

Mechanism of action of CD20 mAbs

Unconjugated CD20 mAbs can exert anti-tumor effect via Fab-mediated and Fc-mediated effects that involve the activation of immune effector mechanisms **Figure 1**. CD20 mAbs can be grouped into type I and type II based on their ability to induce the reorganization of CD20 molecules into lipid rafts upon binding [11]. Type I CD20 mAbs induce the reorganization of

Table 1. Properties of human FcγRs

	FcγRI	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb
Gene	*FCGR1A*	*FCGR2A*	*FCGR2B*	*FCGR2C*	*FCGR3A*	*FCGR3B*
SNPs[1]		H131R	I232T		V158F	NA1, NA2, SH
CNV	No	No	No	Yes	Yes	Yes
Affinity	High	Low to medium	Low to medium	Low to medium	Low to medium	Low to medium
Ligand preference	IgG1 = IgG3 > IgG4	H131: IgG1 > IgG2 = IgG4 R131: IgG1 > IgG3 > IgG4 > IgG2	IgG4 = IgG3 = IgG1 > IgG2	IgG4 = IgG3 = IgG1 > IgG2	V158: IgG3 > IgG1 > IgG4 > IgG2 F158: IgG3 > IgG1 > IgG4 > IgG2	NA1/ NA2/ SH: IgG3 > IgG1
Expression pattern	Macrophages, monocytes, PMNs, eosinophils, dendritic cells	Macrophages, PMNs, eosinophils, dendritic cells, platelets	B cells, mast cells, basophils, macrophages, eosinophils, PMNs, dendritic cells	Monocytes, PMNs, NK cells	Macrophages, monocytes, NK cells, mast cells, eosinophils, dendritic cells	PMNs, eosinophils
Type	Activating	Activating	Inhibiting	Activating	Activating	Activating (GPI-linked)[2]
Signaling	FcRγ-chain ITAM	Own ITAM	Own ITIM	Own ITAM	FcRγ-chain ITAM	Via FcγRIIa or CR3

[1]Only those SNPs are listed that directly influence IgG binding. In case of *FCGR2C, the FCGR2C*-ORF genotype is due to a G > C mutation at position −386 that results in functional expression of FcγRIIc. [2]FcγRIIIb is a GPI-linked membrane protein that is shown to signal via FcγRIIa and / or CR3.

CD20 molecules into lipid rafts and efficiently activate the classical pathway of the complement system. In contrast, type II CD20 mAbs poorly activate complement, but are capable to directly induce cell death upon binding to CD20 without cross-linking by secondary Abs. Both types are capable of inducing antibody dependent cell-mediated cytotoxicy (ADCC) in the presence of effector cells.

Antibody dependent cell-mediated cytotoxicity

A large body of evidence, both from pre-clinical and clinical studies, supports a role for Fc receptors during RTX therapy suggesting that ADCC is an important mechanism of action of RTX.

Fcγ receptors (FcγRs) are surface receptors for IgG and are broadly expressed on leukocytes and varying affinity to the different IgG subclasses (**Table 1**). Functionally they can be grouped into activating and inhibiting receptors depending on the nature of the signal they induce after receptor crosslinking by IgG. Most activating FcγRs associate with the signal transducing γ-chain (FcRγ-chain) [12]. FcRγ-chain is also required for surface expression, illustrated by the lack of surface expression of all activating FcγRs in FcRγ-chain knockout (KO) mice [13]. Cellular activation upon cross-linking of the receptors by IgG-IC is induced by the immunoreceptor tyrosine–based activation motifs (ITAM) that are located on the intracellular part of the FcRγ-chain. The inhibitory FcγRIIb is a single chain molecule that inhibits cellular activation when co-engaged with activating FcγRs. The inhibitory signal is transmitted through the immunoreceptor tyrosine-based inhibiton (ITIM) motif that is located on the intracellular part of the receptor [12].

There is both pre-clinical and clinical evidence that the interaction of the Ab Fc part with cellular receptors is important for the activity of therapeutic CD20 mAbs. Studies in mice demonstrated that CD20 mAb therapy is no longer effective in FcRγ-chain KO mice [14-18]. In contrast to FcRγ-chain KO mice, CD20 mAb therapy was enhanced in mice lacking the inhibitory FcγRIIb in both a xenograft and a syngenic tumor model [14, 16, 17]. In other studies, however, using different tumor models, the inhibitory role of FcγRIIb was not confirmed [18]. This discrepancy most likely caused by the different characteristics of the animal models and the number of FcγRIIb+ effector cells at the tumor site. In mouse tumor models, the IgG2a isotype was found to be the most potent in depleting both normal and malignant B cells. IgG2a efficiently engages both activating FcγRI and

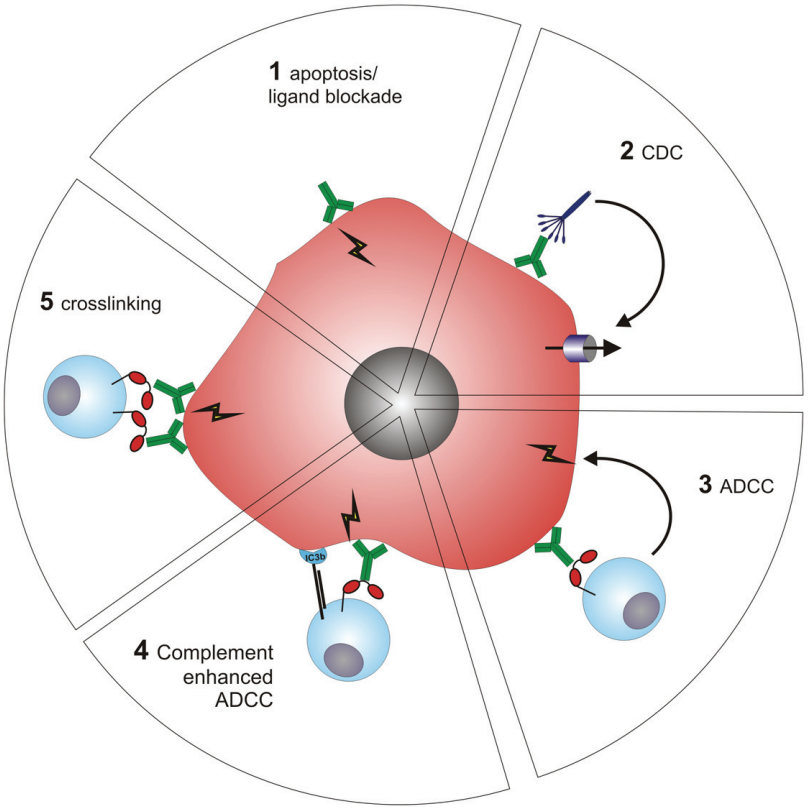

Figure 1. Mechanisms of action of therapeutic CD20 mAbs. CD20 mAbs can induce tumor killing in several ways. A. Direct binding of CD20 mAbs initiate the crosslinking of multiple CD20 molecules, resulting in cell-death via induction of non-classical apoptosis; B. Activation of complement result in complement-dependent cytotoxicity; C. recognition of opsonized tumor cells by FcγRs expressed on immune effector cells initiate antibody dependent cell-mediated cytotoxicity; D. FcγR may only serve as crosslinking platform and thereby enhance antigen signaling in the tumor cells; E. Ab-initiated complement activation yields to deposition of complement cleavage fragments, which may enhance tumor killing through recognition by complement receptors (CRs) in a process called complement-enhanced ADCC.

FcγRIV [15], and has the most favorable A/I ratio [19]. Expression of the intermediate affinity FcγRIV alone was sufficient for the elimination of low tumor burden, whereas elimination of high tumor burden required the presence of all activating FcγR: FcγRI, FcγRIII and FcγRIV [17].

The role of FcγR in the RTX therapy is further supported by clinical association studies performed in RTX-treated individuals. Several single nucleotide polymorphisms (SNP) are described in the genes encoding the low affinity FcγR (*FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, *FCGR3B*). These SNPs either directly influence ligand binding or are located in the promoter region and alter receptor expression [20]. The SNP in *FCGR2C* results in the expression of functional FcγRIIc in 18% of healthy individuals [21]. More recently copy number variation (CNV) have also been described in *FCGR2C*, *FCGR3A* and *FCGR3B* [20, 22]. However, genetic association studies in the complex FcγR region are complicated by the high homology of the FcγR genes.

The different FcγRs have different affinity to IgG subclasses (**Table 1**). In humans, for instance, both IgG1 and IgG3 interact well with all FcγRs and therefore these are potent subclasses capable of efficiently inducing effector functions. IgG2, on the other hand, binds predominantly to FcγRIIa with a lower affinity [23].

Polymorphisms in FcγRs genes can influence the affinity for IgG. FcγRIIIa has two allelic variant that results either in a valine (V) or a phenylalanine (F) at position 158. The V158 allotype binds IgG1 and IgG3 with an increased affinity compared to the F158 allotype [24, 25]. The presence of a homozygous V158 allele in RTX-treated NHL patients was shown to associate with longer progression free survival [26, 27]. An SNP in *FCGR2A* results in an amino acid substitution at position 131 arginine (R) to histidine (H). The H131 variant is able to bind IgG2 [28]. In certain studies this SNP is also associated with RTX therapy [27], which is likely caused by linkage disequilibrium between the *FCGR3A* and *FCGR2A* genes. In CLL however, FcγR polymorphisms did not associate with response with RTX treatment alone [29] or in combination with chemotherapy [30]. The polymorphisms I232T in *FCGR2B* encoding the inhibitory FcγRIIb influence signaling capability through exclusion of FcγRIIb from lipid rafts

[31]. This polymorphism is convincingly shown to be associated with SLE and with parasite infections in humans [32]. Interestingly, *FCGR2B* I232T polymorphisms does not associate with RTX therapy in FL [33]. Although several reports have shown that CNV in FcγRs associate with inflammatory autoimmune diseases [21, 22, 34-36], no CNV in FcγR genes were correlated with outcome of RTX treatment so far.

The nature of the effector cells mediating ADCC

FcγR are expressed on several immune effector cell populations. In *ex vivo* ADCC assays using isolated human FcγR positive effector cells are all able to induce the lysis or phagocytosis of CD20 positive tumor cells in the presence of CD20 mAbs [26, 37, 38]. It is however less clear which effector cell population contributes to *in vivo* efficacy of CD20 mAbs.

Natural killer (NK) cells are potent effector cell population and able to induce tumor cell lysis at low (1:1) effector-to-target (E:T) ratios [39]. The strong association of RTX efficacy with polymorphism in FcγRIIIa, the only FcγR expressed on NK cells, implies a role for this effector cell population [27]. In line with this, stimulating NK cells with an anti-CD137 mAbs *in vitro* and *in vivo* results in enhanced RTX-dependent cytotoxicity [40]. Mouse studies, however, have not found evidence for the role of NK cells [15, 41, 42]. This may be due to the low expression of FcγRIII on mouse NK cells [43]. The observation in mice that FcγRIIb modulates RTX activity *in vivo* suggest that the effector cell type co-expresses both activating and inhibiting FcγR, which questions the role of NK cells. It is also important to note that *in vitro* ADCC assays using human NK cells represent an allogeneic setting and therefore likely overestimate the cytotoxic capacity of NK cells [44].

Polymorphonuclear granulocytes (PMNs) are the most populous FcR positive effector cell type in the blood and their role in mAb therapy is often overlooked. Human PMNs are able to induce efficient cytotoxicity *in vitro* by IgG1 or by IgG2 anti-tumor Abs [45, 46]. PMNs were shown to be contribute *in vivo* to RTX action against B cell lymphoma cells in SCID mice [47]. However, in a mouse model of B cell depletion using human CD20 transgenic mice no role was found for PMNs [41]. This was confirmed by an endogenous B cell depletion model using anti-mouse CD20 Abs in wild type mice [48]. In contrast to mouse PMNs, human PMNs express FcγRIIa, which may result in underestimation of the role of PMNs in mouse studies.

Monocytes / macrophages have been shown to be important for B cell depletion in various mouse model [17, 41]. Our results obtained with a short term peritoneal tumor model, confirmed the predominant role for macrophages [18]. It is still not fully clear in which organ the phagocytic cells reside that mediate depletion of B cells. Interestingly, not splenectomy but compromised blood flow through the liver affected CD20 mAb efficacy [41, 48]. Recently, an important role has been suggested for resident (non-classical) $LyC6^{low}$ monocytes in B cell depletion [48]. Furthermore, tumor cells were found to be associated *in vivo* with macrophages and PMNs during mAb therapy in mice [42].

Most likely the contribution of phagocytic cells to B cell depletion in the different organs is strongly influences by the nature of the tumor or the subset of the B cells that is targeted. Circulatory dynamics of normal – and most likely also of malignant – B cells is also important since circulating B cells are more sensitive to depletion, whereas MZ B cells or peritoneal B1 B cells are more resistant for depletion [41]. When they are mobilized by anti-integrin antibodies they are also efficiently depleted, showing that there is a need to access the circulation [41]. In line with this, blocking CD47 function using an anti-CD47 Ab in a human NHL xenograft model was shown to synergize with RTX action and this was dependent on macrophages and not on NK cells [49]. Macrophages are also found recruited to the tumor site, however, whether they beneficial or not is still a matter of debate [50, 51].

Taken together, there is convincing evidence from both preclinical and clinical association studies for a role for FcγR during RTX therapy. Based on mouse studies, FcγR-expressing monocytes and / or macrophages seem to be most important effector cells. However, it is clear that FcγR-mediated effector mechanisms do not always explain clinical RTX efficacy, indicating a role for other effector mechanisms.

Complement activation

The role of complement in RTX therapy is rather controversial with available data showing beneficial, neutral and even detrimental effects [52]. RTX efficiently binds C1q, activates complement *in vitro* and induce complement-dependent cytotoxicity (CDC) in lymphoma cell lines and primary tumor cells [11, 53].

A number of studies suggest a beneficial role for complement during RTX therapy, often based on the detection of tumor evasion mechanisms from complement-mediated killing. Complement–regulatory proteins (CRP) are frequently observed on circulating tumor cells and complement resistance from complement-mediated lysis of these tumor cells *in vitro* was dependent on the expression of CD55 and CD59 [54]. Overexpression of the CRPs CD55 and CD59 is shown to correlate with resistance in B cell lymphoma cell lines *in vitro* [55-57]. *In vitro* and *in vivo* neutralization of CRPs by Abs increases complement mediated tumor kill and overcomes complement resistance [58, 59]. Furthermore, complement is rapidly depleted after RTX infusion in CLL patients [60] or in mouse models [17]. In line with this, injection of complement-active fresh frozen plasma enhanced B cell depletion [61, 62]. In a mouse study using a syngeneic tumor model expressing human CD20, the first component of the classical complement pathway, C1q, was essential for RTX therapy [63]. Using the same tumor cells we have found that type I CD20 mAbs require complement for elimination of both low and large tumor burden [18].

Other studies, however, question the involvement the complement. In a pre-clinical model for normal or malignant B cell depletion using cobra venom factor (CVF)-depleted mice, or mice deficient for complement factors C1q, C3 or C4 no role for complement was found [15, 17]. Interestingly, in a model using human CD20 transgenic mice CVF treatment affected the depletion of MZ B cells but not circulating B cells [41]. It appears that complement has no role in fully syngeneic mouse models, where the CD20 mAbs used do not cross-react with the normal host B cell reservoir [64].

In humans, expression of CRPs on tumor cells did not predict clinical outcome in FL [65] or in CLL [66]. Furthermore, it was proposed that that complement activation inhibits ADCC by NK cells as a results of deposited iC3b on tumor cells that blocks binding of IgG to FcγRIIIa [67]. This was also confirmed in an *in vivo* model [68]. In line with this, a polymorphism in C1qA associated with low C1q levels correlated with better response to RTX therapy in FL [69]. The most often observed adverse effects during mAb therapy is infusion reactions (fever and / or chill and skin rashes etc), which is most pronounced at the first infusion may be attributed to complement activation [70].

Next to inducing direct tumor lysis by CDC, complement may play additional roles during RTX therapy. Deposited complement fragments (C3b(i)) can be detected upon incubation of CLL cells with RTX *ex vivo* [71] and also on tumor cells in mouse models [18]. These fragments can serve as ligands for complement receptors (CRs) expressed on effector cells, such as macrophages. Binding of iC3b to CR3 alone can result in direct complement-dependent cellular cytotoxicity (CDCC). This mechanism requires CR3 to be preactivated and therefore unlikely to contribute to the mAb therapy under normal circumstances [72].

There is interplay between FcγR and complement. During complement activation small cleavage fragments C3a and C5a are generated that function as anaphylatoxins to further recruit effector cells to the site of the tumor. Binding of C5a to C5aR on local macrophages shifts the balance of activating and inhibitory FcγR expression resulting in activated state [73]. Moreover, CR3 binding to iC3b ligands can also serve to amplify ADCC. mAb therapy of large tumor burden by type I CD20 mAbs was dependent on CR3 expression in a peritoneal tumor model. In line with this, we have found that in *in vitro* macrophage-mediated killing assays iC3b-CR3 interactions tipped the balance of killing at suboptimal E:T ratios [18].

The second FDA-approved unconjugated CD20 mAb OFA activates complement more efficiently than RTX, presumably because it binds a unique, membrane proximal epitope compared to RTX [55, 74]. OFA is able to lyse even RTX-resistant Raji cell line expressing low levels of CD20 [74], primary tumor cell lines resistant to RTX *in vitro* [55] or in a mouse model of human lymphoma [75]. Whether this better complement activation translates into superior clinical

efficacy in head-to-head trials with RTX remains to be seen.

Taken together, the role of complement is currently controversial and requires further investigations. It is likely that next to CD20 expression levels and CRP expression levels on the tumor cells, circulatory dynamics of normal or malignant B cells, their anatomical location or tumor burden, together influence the involvement of complement in CD20 therapy.

Apoptosis induced via cross-linking of RTX by FcR-expressing cells

It has been suggested that RTX induce apoptosis of malignant B cells, based on increased apoptosis in tumor cells from CLL patients treated with RTX compared to untreated patients [76]. This was supported by *in vitro* studies; where RTX was cross-linked by a secondary Ab or by FcR-expressing cells [77, 78]. It is therefore possible that *in vivo* FcR-expressing cells would provide cross-linking stimulus similar to secondary Abs *in vitro* inducing cell death in the tumor cell. It has been shown that the *in vivo* activity of the agonistic CD40 mAb depends on cross-linking provided by FcγRIIb demonstrating that cross-linking by FcγRs can be an important mechanism of action of mAb therapy [79, 80]. For CD20 mAb therapy, expression of FcγRs are needed as shown by FcRγ-chain KO mice, but whether this is required for active signaling or whether FcγR are purely serve as a scaffold to provide cross-linking was unclear. To investigate if cross-linking by FcγR *in vivo* possibly contributes to the mechanism of action of CD20 mAb, a novel transgenic mouse strain (NOTAM) was generated. NOTAM mice carry a mutation in the ITAM of the FcRγ-chain and have normal surface expression of activating FcγR [81]. Using the NOTAM mice we demonstrated that cross-linking by FcR does not contribute to *in vivo* action of type I CD20 mAbs [81]. Our finding was confirmed in a different model using BJAB tumor cells and mice that only express FcγRIIb [82]. Therefore current data suggest that cross-linking by FcγR does not substantially contribute to the *in vivo* mechanism of action of CD20 mAb.

Fab-mediated direct induction of cell death

In contrast to type I, type II CD20 mAb or tositumumab-like reagents are able to induce direct cell death upon binding to CD20 [83, 84]. This is independent of the Fc part of the Ab but requires $F(ab)_2$ fragments and the binding of at least two CD20 molecules [85, 86]. The induction of programmed cell death (PCD) follows a mechanism that is independent of BCL-2 and caspases but requires lysosomal enzymes [83, 87]. More recently it was shown that cell-death evoked by type II CD20 mAbs requires a novel reactive oxygen species-dependent pathway [88].

The prototypic type II CD20 mAb B1 is shown to exert superior effects when compared with type I CD20 mAbs in preclinical model of B cell depletion [37]. This was confirmed with GA101 (obinutuzumab), another clinical candidate type II CD20 mAb in a xenograft tumor model as monotherapy [89] or in combination with chemotherapy [90]. GA101's ability to induce apoptosis and its Fc-independent mode of action has potentially important advantage over RTX in circumventing tumor cell resistance to apoptosis or avoid influence of exhaustion of FcR-bearing effector cells.

Induction of long-term anti-tumor immunity

Next to the direct anti-tumor effects, CD20 mAb may induce long-term anti-tumor immunity, which could be the underlying reason why some patients experience durable tumor regression [91]. Ab-opsonized tumor cells may be phagocytosed by tissue resident dendritic cells (DCs) that can induce the generation of tumor-specific cytotoxic T cells [92]. This idea is supported by the observation that in patients, clinical tumor shrinkage is only visible after one week after the first infusion. Pre-clinical evidence shows that CD20 mAbs induce protective anti-tumor responses against human CD20-expressing tumors in immunocompetent mice [93]. Immune complexes (ICs) that are formed during CD20 mAb therapy are potent activators of DCs and could induce anti-tumor immunity [94-96]. ICs are effectively taken up by DCs via activating FcγRs, which leads to potent stimulation of DCs culminating in cross-presentation of tumor-derived antigens [96]. Therefore it cannot be excluded that the association between FcγR polymorphisms in RTX-treated patients reflects a role for FcγR on DCs may be involved in inducing anti-tumor immunity [92]. Currently, chemotherapy is given together with CD20 mAbs. Preclinical studies with herceptin showed that chemotherapy may

impair the developing tumor-specific T cell response [97]. This may also be true for CD20 mAb therapy, suggesting that altering the treatment schedule could allow the generation of adaptive anti-tumor responses.

Synergy of effector mechanisms

It is likely that *in vivo* a combination of effector mechanism is involved in anti-tumor action of CD20 mAbs. The dominant effector mechanism may change during the course of the therapy and perhaps also differ between different anatomical locations. For instance, circulating tumor cells can be eliminated by complement in blood or via the phagocytic cells of the liver or spleen. Killing of a non-circulating tumor cell in an anatomical compartment, where complement levels are relatively low, may rely on other mechanisms.

Tumor burden may also impact effector mechanisms of CD20 mAbs. We showed in a short peritoneal tumor model that complement activation is sufficient for the elimination of low tumor burden. However, elimination of high tumor burden by CD20 mAb requires a concerted action of all effector mechanisms, because lack of FcγR, complement or CR3 expression fully abrogated therapy [18].

RTX and chemotherapy

RTX works synergistically with chemotherapy in the clinic [98]. The mechanism likely underlying this synergy is that RTX sensitizes lymphoma cells for chemotherapy by downregulating anti-apoptotic proteins [99-101]. Using a type II CD20 mAb GA101, it was shown *in vitro* that CD40 stimulation of peripheral CLL cells sensitizes tumor cells for direct cell death induction [102]. These results suggest that combination of cytotoxic drugs with CD20 mAb can be beneficial.

Resistance to RTX therapy

The majority of the cancer patients eventually become refractory to RTX treatment. There are several mechanisms by which this can happen, including increased Ab catabolism, selection of tumor cells with low-levels of surface CD20 expression, resistance to mAb-effector mechanisms or impaired immune cell function [103]. An important escape mechanism is the loss of CD20 from tumor cells after RTX treatment [104]. It was recently shown that type I CD20 mAbs induce internalization of CD20 by tumor cells. The degree of internalization is higher with type I than with type II CD20 mAbs [105]. The internalization requires the binding of the Fc tail of RTX to FcγRIIb on the same tumor cell. In support of this, FcγRIIb expression on different forms of B cell malignancies was correlated with CD20 internalization and with resistance to RTX therapy [106].

CD20 can also be removed from the surface of the tumor cells in a process initially described as shaving [60, 107]. Shaving of CD20 follows the cellular mechanisms of trogocytosis. Trogocytosis was first described during antigen presentation between T cells and DCs and involves transfer of cell membrane between two cells during immunological synapse formation. Similarly, cytotoxic synapses are formed during ADCC and CD20 molecules are transferred from the surface of the tumor cells to the effector cells and subsequently internalized. Trogocytosis of CD20 results in CD20-negative B cells, which are therefore resistant to CD20 mAb therapy. Trogocytosis of CD20 requires Fc receptors, such as human FcγRI [107]. We showed in a mouse model that all mouse FcγR, both activating and inhibiting FcγR are able to mediate trogocytosis of CD20 [108]. Whether internalization or trogocytosis of CD20 is more relevant *in vivo* mechanism for CD20 mAb therapy will need to be further investigated. A recent report suggest, however, that trogocytosis occurs faster then internalization [109].

Novel CD20 mAbs

Second generation CD20 mAbs carry a humanized or fully human and are expected to be less immunogenic than the chimeric Ab RTX [110]. Examples are ofatumumab, the humanized ocrelizumab [111] and veltuzumab [112]. Ofatumumab (OFA, Arzerra) was the second unconjugated CD20 mAb that has been approved by the FDA [113]. OFA is a fully human CD20 mAb recognizes an overlapping epitope on the small and big extracellular loop of CD20, which results in better complement activation than RTX [55, 114]. Veltuzumab (IMMU-106, hA20) is a humanized CD20 mAb that carry similar variable sequences to RTX but expected to induce stronger CDC than RTX [110]. Ocrelizumab (Genentech / Roche) is a human-

ized CD20 mAb with increased binding affinity for the low-affinity variants of the FcγRIIIa receptor [110].

Third generation CD20 mAb have improved effector functions by glyco- or protein-engineering and include obinutuzumab, TRU-015, AME133V and Pro131921. Obinutuzumab (GA101, GlycArt / Roche) is the most advanced of these reagents. In contrast to all other CD20 mAbs that are in clinical development, obinutuzumab is unique in that it is a type II CD20 mAb. Pre-clinical studies suggest, that type II CD20 mAb may outperform type I CD20 mAbs both in xenograft tumor studies [85, 90] and in depletion of normal B cells [37]. This may partially be due to the fact that type II CD20 mAbs induce less CD20 internalization than type I CD20 mAbs [105]. Obinutuzumab was selected for its superior ability to induce cell death in tumor cells [89]. In addition to its improved ability to induce cell death, the Fc region of obinutuzumab is glycoengineered to provide better binding to FcγRIIIa independent of its allotype [115]. It will be difficult to separate the advantage provided by these two modifications, however, preclinical studies using a non-glycoenginered version of obinutuzumab suggest that its type II characteristics are more important [116]. Obinutuzumab was well tolerated in Phase 1 clinical studies [117, 118]. AME-133v (LY2469298) and PRO131921 are both humanized type I CD20 mAb with protein-engineered Fc part that provide enhanced affinity for FcγRIIIa and therefore induce better ADCC activity compared to RTX [110]. TRU-015 is a smaller CD20 mAb with retained Fc-mediated effector functions and is currently in development for RA [110].

Future directions

Despite intense research, the mechanisms of action of CD20 mAb are still incompletely understood [119]. There are several novel, next generation CD20 mAb currently in development or under clinical testing phase [120]. The results and efficacy data from these studies will inevitably learn us about the mechanisms of CD20 mAbs in patients.

Exhaustion of effector mechanisms during CD20 mAbs therapy can be a problem [121]. Lack of available active complement can be circumvented by injection of fresh frozen plasma. It was shown that much lower dose of RTX that is currently given may also been effective in eliminating tumor cells [61]. To prevent exhaustion of immune effector mechanisms the CD20 mAb dosing should be revisited. Altered timing of chemotherapy and mAb therapy may allow the generation of long-term protective anti-tumor immunity.

Research in the recent years has identified several mutations of combinations of mutations that modify IgG affinity to different FcγR [122]. In addition, our understanding on how glycose side-chains influence IgG properties have also increased [123]. Mutations introduced in the CH3 domain of IgG1 affecting binding to FcRn, which confer to longer serum half-life, will possibly allow less frequent administration of drugs [124]. Protein- and / or glyco-engineering of therapeutic Abs promise to be a powerful strategy to improve their efficacy.

We need to better understand the impact of FcγR polymorphisms on CD20 mAb therapy, how SNPs and CNVs influence therapeutic efficacy in different B cell malignancies. Patients may be pre-screened in the future for FcγR polymorphisms and expression of FcγRIIb on tumor cells may be determined. Patients with low FcγRIIb expression may be treated with type I CD20 mAbs, whereas patients with high FcγRIIb expression may benefit from the use of antibody drug-conjugates (ADCs), where internalization of the target is desirable. Protein- and / or glyco-engineering of Fc part of the Abs can influence the binding for FcγRs. Type I CD20 mAbs engineered to bind activating FcγR more efficiently and / or FcγRIIb less efficiently may provide better therapy. Better insight into the mechanisms of action of CD20 mAbs together with novel screening methods will allow the selection of the optimal treatment regimen.

In addition, combination of RTX with CD47 can enhance phagocytosis [49]. Double targeting or of CD20 and CD47 with bispecific Abs was shown to induce efficient apoptosis in primary malignant cells [49, 125].

Conclusions

Currently, several novel CD20 mAbs, improved in different ways are in clinical testing phase or close to obtain regulatory approval. The coming

years will learn us whether these improvements will also translate to increased clinical efficacy. The success or failure of these novel Abs will inevitably generate better insight into the mechanism of action of CD20 therapeutic mAbs.

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Disclose of potential conflict of interest

None.

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