CD20 as a Target for Therapeutic Type I and II Monoclonal Antibodies

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The last decade has seen the monoclonal antibody (mAb), rituximab, transform clinical management of many non-Hodgkin lymphomas and more recently provide new opportunities for controlling autoimmune conditions, such as rheumatoid arthritis. Although not yet fully determined, the explanation for this success appears to lie with the inherent properties of its target, CD20, which allow rituximab to recruit potent cytotoxic effectors with unusual efficiency. In this review we detail the properties of CD20 that make it such an effective therapeutic target and describe how different mAbs change the membrane distribution and internalization of CD20 and have distinct modes of cytotoxic activity.

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CD20 was first identified as a B-cell marker in 1980 by Stashenko et al., who hypothesized that “a phenotypic marker with representation limited to B lymphocytes would therefore be extremely useful in the enumeration, fractionation, and analysis of function of the B cell population.” We now know that the CD20 molecule, originally identified as B1, is expressed on early pre-B cells, remains through B-cell development, and is then lost from plasma cells. Most importantly for its monoclonal antibody (mAb)-targeting success, it is expressed on the majority of B-cell lymphomas.

CD20 would probably have attracted little academic attention had it been no more than a marker for identifying B cells. Instead, it has provoked intense study based on its status as an as yet unparalleled target for mAb immunotherapy. The characteristics that make it such a good target antigen are still disputed but likely involve its relatively high level of expression and the fact that the extracellular epitopes recognized by the various mAbs are close to the cell surface, attributes that facilitate high levels of mAbs near the cell surface and thus permit efficient engagement of Fc-dependent effector mechanisms.

CD20 STRUCTURE AND REGULATION

CD20 is a nonglycosylated member of the membrane-spanning 4-A (MS4A) family that includes at least 26 proteins in mice and humans. Hydrophaticity analysis of its sequence predicted three hydrophobic regions forming a tetraspan transmembrane molecule with a single extracellular loop and intracellular N- and C-terminal regions. Although no crystal structure has been obtained, these early topology predictions have been largely confirmed by proteolytic studies. Furthermore, the availability of recombinant protein through bacterial expression and its use in reconstitution experiments has shown that in accordance with the predicted structure, the majority of the molecule is alpha helical and very stable to denaturants. The greatest sequence conservation between members of the MS4A family is observed in the hydrophobic regions, suggesting that these and the resulting tetraspan structure are probably central to their function. The intracellular regions of CD20 contain numerous sites for serine and threonine phosphorylation, which account for the different isoforms, ranging from 33 to 36 kDa, detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Importantly, phosphorylation of these residues has been shown to be occur during cell cycle progression and proliferation in response to B-cell antigen receptor (BCR) engagement and phorbol ester stimulation and to increase with direct ligation by anti-CD20 mAb.

ANTIBODY BINDING

On the cell surface CD20 is predicted to display a loop of approximately 40 amino acids between the proposed third and fourth transmembrane helical do-
mains, with the potential for a second far smaller loop located between transmembrane domains 1 and 2. The length of this putative small loop is highly conserved between members of the MS4A family, and if it is present it would be formed from within the first section of hydrophobic residues. The evidence for the existence of a small loop at the membrane surface is somewhat contradictory. Julie Deans’ group demonstrated that in intact cells there was no protease digestion of CD20 other than that observed in the large loop. This resistance to digestion of the proposed smaller loop was seen even when using proteinase K, a relatively promiscuous enzyme that should be able to cleave fragments as small as four amino acids in length. In apparent contradiction to these observations, our group working in collaboration with Genmab found that some anti-CD20 mAbs recognize peptide sequences corresponding to the region of the small loop, but it is important to note that these studies have not been corroborated by the mutational analysis used to confirm the surface expression of the larger loop. Although these observations appear contradictory, they are not irreconcilable, as recent crystallographic studies by the groups of Guo and Ding have shown that the proposed antigen binding site for the anti-CD20 mAb ofatumumab (the clinical version of the 2F2 mAb) contains a ring of hydrophobic residues surrounding a deep binding cleft with a positively charged interior. They postulate that this highly hydrophobic interface could enable ofatumumab to interact with and indeed penetrate the membrane surface, permitting more extensive binding to the small loop. This unusual property could confer on ofatumumab, and other related anti-CD20 mAbs (7D8, 11B8), their strikingly slow off-rates. Beyond these few exceptions, the larger loop appears to provide the epitopes for binding of other anti-CD20 mAbs generated to date, with alanine 170 and proline 172 being the most critical residues for binding. Recent evidence from recombinant expression and crystallography studies has demonstrated the importance of a disulfide bond between cysteines 167 and 183 for the efficient binding of rituximab, most likely through the maintenance of a loop structure and retention of its conformational epitope. Although this cysteine bond has not been investigated for its effects on the biological function of CD20, it is tempting to speculate that with more than half of the MS4A family predicted to possess a disulfide bond in the large extracellular loop, this feature will prove to be of importance for the maintenance of the extracellular structure and perhaps self-multimerization of this family of molecules (see below).

Using various techniques, including immunoprecipitation following cell lysis in different detergents and co-immunoprecipitation with tagged and truncated forms of the molecule, CD20 has been shown to be present on the cell surface as homo-multimer, likely tetramer, complexes. Confocal and electron microscopy have demonstrated that approximately 90% of this complexed CD20 is constitutively associated with lipid rafts and microvilli. Indeed, some anti-CD20 mAbs, such as FMC7, will only bind when CD20 is in rafts and others, including rituximab, increase raft association of CD20 through epitope-dependent but cross-linking-independent events. In addition to its presence as a homo-multimer, CD20 has been shown by immunoprecipitation and fluorescence resonance energy transfer (FRET) studies to associate with numerous other molecules, many of which are enriched in lipid rafts and dependent on rafts for signaling, such as major histocompatibility complex (MHC) class I, MHC class II, CD53, CD81, CD82, CD40, and, perhaps most notably, the BCR.

CD20 FUNCTION

Despite nearly 30 years of intensive study, as yet no ligand has been revealed for CD20, and until very recently its function remained obscure, with CD20-deficient mice displaying only subtle phenotypes. Consequently, when increased calcium conductance was observed in cells transfected with CD20, it was proposed that CD20 itself functioned as a calcium ion channel, although direct evidence was lacking. This idea was supported by subsequent data showing that, through its association with the BCR in lipid rafts, CD20 is involved in store-operated cation (SOC) entry following BCR ligation, implying that it plays a role in regulating cytoplasmic calcium levels after antigen engagement. Further to this, in experiments using a variety of inhibitors and with cells expressing different levels of CD20, we recently demonstrated that the calcium flux (together with activation of ERK1–2, BLNK, and SLP76) elicited by the engagement and further cross-linking of CD20 is itself dependent on BCR expression, indicating that CD20 signals generated after mAb ligation occur largely through its association with and “hijacking” of the BCR signaling pathway. These observations are consistent with elegant work by Deans and coworkers, who in extensive immunoprecipitation and FRET experiments have demonstrated that CD20 is physically associated with a subpopulation of the cell surface BCR and that this association is lost following BCR signaling. Subsequently, CD20 transiently associates with phosphoproteins and calmodulin-binding proteins, indicating its role in the propagation of BCR signaling events. These associations with the BCR are supported by the stage-specific expression of CD20 on B cells where it is found to just precede and then parallel the expression of surface IgM, and by the observation that in CD20-deficient mice there is a small reduction in BCR expression together with a reduction in both BCR and CD19 ligation-induced calcium responses.
Taken together, these various studies point to the biological role of CD20 as an amplifier of calcium signals that are transduced through the BCR during antigen recognition by immature and mature B cells. However, regardless of its true function in B cells, interest has mainly focused on CD20 as a target for mAb immunotherapy.

**CLASSIFICATION OF ANTI-CD20 mAb**

The very earliest in vitro studies comparing two isotype-matched anti-CD20 mAbs, 1F5 and B1 (tositumomab), demonstrated that not all anti-CD20 mAbs are the same.\(^{28}\) These studies showed that 1F5 but not B1 could stimulate resting B cells to enter the cell cycle and to proliferate in response to growth factors. In marked contrast, both mAbs inhibited immunoglobulin secretion by activated B cells. As the number of anti-CD20 mAbs increased, various attempts were made to classify them according to either their binding or functional properties. In the first of these, four different groups of mAbs were identified based on their ability to bind to a chimeric CD20 molecule containing the large extracellular loop from mouse CD20 but with the critical binding AxP motif taken from human CD20.\(^{13}\) The four groups were: group I, containing just one mAb (CAT 13.6E12), which was unable to recognize this construct; groups II and III, which bound at low or intermediate levels; and group IV, which contained the majority of mAbs (including rituximab and B1), all able to bind strongly to the chimeric construct. In later studies we identified a fifth group of fully human mAbs that did not require the AxP motif and whose binding also appeared to involve the small loop described above.\(^{12}\) However, functionally, these five different groups of anti-CD20 mAbs can be divided into just two distinct subtypes, which we term type I and II.\(^{29}\) Type I (rituximab-like) mAbs induce CD20 to redistribute into large detergent resistant microdomains (rafts), whereas type II (tositumomab-like) mAbs do not. This differential ability of anti-CD20 mAbs to redistribute CD20 in the plasma membrane had been identified previously by Deans et al.\(^{30}\) Importantly, we now know that this redistribution impacts on many of the binding properties and effector functions that control the therapeutic success of anti-CD20 mAbs.

The majority of anti-CD20 mAbs generated to date are type I (reviewed by Lim et al\(^{31}\)) and they display a remarkable ability to activate complement and elicit complement-dependent cytotoxicity (CDC) through enhanced recruitment of C1q. This activity appears to be directly linked to their translocation into lipid rafts, which cluster the antibody Fc regions thus enabling improved C1q binding.\(^{32}\) Type II mAbs, in marked contrast, do not appreciably change CD20 distribution, and without the concomitant clustering, they are relatively ineffective in CDC. Intriguingly, however, they evoke far more homotypic adhesion and direct killing of target cells\(^{33-35}\) (see below). Finally, both type I and II mAbs appear to demonstrate efficient phagocytosis and antibody-dependent cytotoxicity (ADCC). The current challenge in the field, therefore, is to reveal the structure function relationship that explains how these two types of mAbs can engage CD20 to evoke such different levels of CDC and direct cell death while maintaining equivalent Fc:FcR-dependent functions, a task made all the more difficult by the apparent similarity in binding site (AxP) for most of these mAbs.

One clue may be in the differential binding stoichiometry of type I and II mAbs, as cell surface CD20 provides only half as many type II epitopes as it does type I epitopes.\(^{32}\) We have previously speculated that this consistent 2:1 ratio may be due to type II mAbs binding within a CD20 tetramer and type I binding between adjacent tetramers.\(^{36}\) However, new structural information provided by the co-crystallization of anti-CD20 Fab fragments with the extracellular binding loop of CD20 and recent data from Glycart (Schlieren, Switzerland)\(^{37}\) using SIDEC’s (Stockholm, Sweden) proprietary protein tomography technology may help to clarify the crucial differences between type I and II mAbs. In particular, the protein tomography data illustrate clear conformational differences in CD20 induced by binding of type I or II mAbs, which suggest that type I mAbs induce CD20 to adopt an “open” configuration likely linked to its role as a SOC calcium channel, whereas type II mAbs, which do not induce a rapid calcium flux, leave the molecule in a “closed” configuration. In keeping with observations that at least some CD20 is constitutively associated with the BCR\(^{16}\) and lipid rafts\(^{17}\) before mAb ligation, CD20 is found to be present in both open and closed states.\(^{37}\) Looking forward, it is hoped that manipulating the lipid rafts and/or signaling molecules in the absence or presence of type I/II mAb binding prior to protein tomography may help to elucidate the key structure/function relationships of anti-CD20 mAbs.

**TYPE II–MEDIATED CELL DEATH**

In addition to the recent progress in understanding of how type I and II mAbs might engage CD20 differently, as detailed above, recent work has shown how type II mAbs can induce a unique mode of cell death. For many years it has been appreciated that rituximab and other type I anti-CD20 mAbs can mediate direct cell killing particularly when hyper-cross-linked by anti-antibody or by the use of mAb multimer conjugates.\(^{38,39}\) This process is somewhat controversial but often bears hallmarks of classical apoptosis and appears largely caspase-dependent; however, it is not clear whether CD20 mAb cross-linking in vivo, for example by FcyR, could ever deliver this form of killing. In contrast, type II mAbs can kill cells without hyper-cross-linking and in a caspase-independent manner.
ing a range of B-cell lines and primary chronic lymphocytic leukemia (CLL) samples, we have now shown that type II mAb-induced cell death is dependent on homotypic adhesion, requires cholesterol, and is energy-dependent, involving the relocalization of mitochondria to the vicinity of cell–cell contact. Furthermore, fluorescence imaging coupled to inhibitor studies revealed that peripheral actin redistribution was required for both adhesion and death, with cell contact areas demonstrating very high levels of reorganization, and with the kinetics of cell death paralleling the adhesion process. Cell death was confirmed to be caspase-independent in support of previous work and not dependent on the key autophagic mediator Beclin-1, instead requiring lysosomes. These lysosomes were shown to swell and release their contents into the cytoplasm and extracellular space, possibly aided by a process of cell membrane exchange coincident with the initial adhesion. The death induced could be blocked, at least partially, by cathepsin inhibitors, further implicating cytoplasmic lysosome products. Importantly, this cytoplasmic cell death is not regulated by anti-apoptotic proteins such as Bcl-2, indicating that it can bypass the normal tumor survival mechanisms that confound conventional chemotherapy treatments. Although this novel form of mAb-mediated cell death was also strongly induced by anti-human leukocyte antigen (HLA)-DR mAbs, but not a variety of other mAb specificities able to induce homotypic aggregation, death with anti-CD20 mAb was not dependent on HLA-DR expression. However, it is still unclear which proximal events trigger the process of actin redistribution and lysosomal release following type II mAb binding, or importantly, what role, if any, it might play in their therapeutic activity.

**IN VIVO MECHANISM OF ACTION OF TYPE I AND II mAb**

Despite a wealth of in vitro data demonstrating differences between type I and II mAbs in various in vitro assays, the importance of these characteristics in vivo has been less clearly defined. In early xenograft experiments, we demonstrated that both type I and II mAbs could provide excellent protection against tumor growth but that their mechanisms of action differed markedly. Using cobra venom factor (CVF) to deplete complement activity, type I mAbs (rituximab and 1F5) were shown, by us and others, to be dependent on complement for much of their in vivo efficacy. In contrast, the type II mAb tositumomab (B1) was unaffected by CVF administration and was able to generate significant tumor protection through an Fc-independent mechanism, presumably the caspase-independent lysosome-mediated cell death described above.

Since then, refined syngeneic models of normal B-cell depletion using either anti-mouse CD20 or anti-human CD20 mAbs in transgenic (Tg) mice (Beers et al, unpublished observations) have been developed. Consistently, these models have shown that all anti-CD20 mAbs, including type I and II reagents, are dependent on macrophage-mediated Fc:FcR interactions and that neither complement nor direct cell death (Beers et al, unpublished data) plays a prominent role in depletion. These observations run counter to the earlier studies with xenografts and likely reflect differences in both the models and reagents, and perhaps a difference between malignant and normal B cells. For example, it is clear with hindsight that depletion of relatively low numbers of xenogeneic tumor cells derived from an extensively passaged cell line may not be a good model for either depletion of large numbers of primary B cells embedded in their correct niche within the body, or indeed malignant B cells localized to particular secondary lymphoid organs. In fact, contrary to our initial suggestions regarding complement, recent evidence from several sources appears to indicate that complement activation may serve to inhibit the activity of type I mAbs such as rituximab. Thus at this point, the role of complement as an effector for anti-CD20 mAb remains controversial, and it is probably safest to believe that while it may not be a major player for removing normal B cells and may inhibit some effectors, it is likely that against particular B-cell types or in certain tissues it will play a role in cell elimination or effector cell activation.

Regarding the current evidence indicating the lack of a role for directly induced cell death with type II mAbs in vivo, this may simply reflect the fact that, in contrast to malignant cells, Tg mouse B cells, like normal human B cells, are relatively resistant to direct killing. In support of this, recent work on a novel type II mAb, GA101, demonstrates that little cell death is induced in normal B cells in the absence of mitogenic stimulation. To date, type II anti-human CD20 mAbs have not been used in a suitable syngeneic malignant tumor model with actively proliferating cells that might prove to be more susceptible to the direct induction of death demonstrated in cell lines. However, anti-mouse CD20 mAbs have been applied to treating malignant cells in a highly aggressive Eμ-myc lymphoma model with limited success, although the anti-mouse CD20 mAbs used were not defined as type I or II and direct death induction was not extensively investigated as a potential mechanism. Regarding this point, it should be noted that anti-mouse CD20 mAbs have not been classified into type I/II subgroups as yet. Mouse CD20 also lacks the key redistribution motifs present in human CD20 and so it remains a possibility that it does not respond to mAb ligation in the same way as the human molecule. Therefore, although direct cell death with type II anti-human CD20 mAbs has not been demonstrated in the available in vivo models, we cannot preclude this mechanism from being important in
less aggressive Tg models or indeed patients, in particular those with impaired effector mechanisms or elevated chemoresistance.

**MODULATION: A NEW NEGATIVE REGULATOR**

Given that direct cell death does not appear to be evoked by type II mAbs in the hCD20 Tg model, and that complement activity is neither required nor detrimental, our recent results using isotype-matched mAbs\(^4^6\) demonstrating that type II mAbs are more potent than their type I counterparts require further explanation. Using a variety of approaches, including analyzing cells from mice lacking activatory Fc\(\gamma R\) and therefore unable to deplete targeted B cells, we made the unexpected observation that type I, but not type II, mAbs induce substantial modulation of CD20 from the B-cell surface (Figure 1; Beers, unpublished data). This antigenic modulation by type I mAbs, which can be measured in vitro and in vivo, has two related consequences: first, the loss of surface mAbs reduces Fc:FcR-mediated effector cell clearance of B cells by approximately fivefold; and second, active removal of mAbs from the circulation reduces its half-life and the longevity of depletion. Although there have been previous reports demonstrating CD20 internalization with mAb ligation,\(^5^2,^5^3\) the majority of studies have shown that CD20 does not modulate. In fact, the first clinical use of anti-CD20 mAb, 1F5, by Press and colleagues\(^5^4\) showed that mAbs did not undergo antigenic modulation from patient tumor cells and this was highlighted as a benefit of such a reagent. Furthermore, we have reported that CD20 did not substantially modulate from a group of primary CLL samples in a short-term 2-hour culture.\(^5^5\) In light of these discrepancies and our recent data in hCD20 Tg mice, we have revisited this issue in human tumors using a highly sensitive modulation assay adapted from the work of Austin et al.\(^5^6\) Using this assay, which quantifies simultaneously the amount of mAb on the cell surface and in the cytoplasm, we find that human cells are highly heterogeneous in their ability to internalize CD20 when bound by type I mAbs. Normal B cells, like those from hCD20 Tg mice, modulate uniformly and rapidly, while malignant cells show a more mixed profile. At one extreme follicular lymphoma, diffuse large B-cell lymphoma, and most B-cell lines maintained in culture tend not to modulate, while over a period of a few hours CLL cells and mantle cell lymphoma are much more prone to internalize a sig-

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**Figure 1.** Type I, but not type II, mAbs induce substantial modulation of CD20 from the B cell surface. By using gamma chain \(-/-\) mice lacking activatory Fc\(\gamma R\) and therefore unable to deplete targeted B cells, we were able to explore the consequence of type I and II anti-CD20 mAb ligation on B cells in vivo in the absence of depletion. (A and B) Differentially carboxyfluorescein succinimidyl ester (CFSE)-labeled human CD20 Tg and wild-type (WT) splenocytes were transferred into gamma chain \(-/-\) recipients and then mAbs (isotype-matched versions of rituximab [Ritm2a], tositumomab [Tosit], or irrelevant [Irr]) injected on day 1. On day 2 splenocytes were stained for B cells with anti-CD19 mAb, and with reagents to detect surface CD20. These experiments revealed that type I mAbs substantially reduced the expression of CD20 on the target cells (~90% loss), whereas type II mAbs had a far less profound effect (~20% loss). (C) Type I (rituximab-like) anti-CD20 mAbs also modulate CD20 from the cell surface of circulating B cells in hCD20 Tg mice. hCD20 Tg x gamma chain \(-/-\) mice were treated with irrelevant IgG, Ritm2a or Tosit, and peripheral blood B cells analyzed 2 days later for the expression of surface CD20; the filled histogram shows background staining.
significant proportion of their surface CD20 (Beers et al, unpublished data). We consider that the results from this sensitive assay and the natural innate heterogeneity between CLL samples may explain our previous observations. Thus, modulation is not confined to normal mouse and human B cells and, intriguingly, we find that those B-cell malignancies where rituximab has proved relatively ineffective as a treatment, ie, CLL and mantle cell lymphoma, had a much greater tendency to modulate. We believe that these findings must be factored into any explanation of treatment outcome and should be added to the growing list of reasons for treatment failure, which includes low CD20 expression, mAb shaving, and mAb consumption. With regard to the phenomenon of CD20 shaving (see Taylor and Lindorfer in this issue of *Seminars*), although we have not formally studied its involvement, it seems unlikely to account for the observed differences between type I and type II mAbs, as it would be expected to effect both to a similar extent.

Based on our observations detailed above, we hypothesize that type I mAb-mediated CD20 modulation and antibody consumption are key factors regulating the therapeutic variations between these diseases and at the heart of differences between type I and II mAbs. We are currently investigating the molecular mechanisms behind this modulation with the anticipation it will relate to the ability of type I but not type II mAb to redistribute CD20 into lipid rafts and associate with the BCR.

**CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS**

Taking all of these data together, it is clear that type I and II mAbs have the ability to evoke different effects both in vitro and in vivo: type I mAbs engage CDC and ADCC and cause modulation in certain B-cell malignancies but do not elicit direct cell death, whereas type II mAbs mediate direct cell death (in certain target cells) and engage ADCC but do not promote CDC or CD20 modulation (Figure 2). The question now is which of these mechanisms are critical in the treatment of human disease, including autoimmunity and B-cell malignancies, and whether these will vary according to the disease in question. For example, will diseases such as follicular lymphoma and diffuse large B-cell lymphoma, which do not undergo rapid modulation upon rituximab binding, benefit from the ability of type I mAbs to activate complement? Conversely, in CLL, will type II mAbs perform better through their ability to elicit direct cell death and avoid modulation with the consequent reduction in mAb consumption but at the expense of having only half as many mAb molecules capable of binding? Furthermore, in addition to simple distinctions between type I and II mAbs, we are now

![Figure 2](image-url)

**Figure 2.** Different effector mechanisms of type I and II anti-CD20 mAbs. Type I mAbs are able to engage CDC and ADCC and cause modulation in certain B-cell malignancies but do not elicit efficient direct cell death, whereas type II mAbs can mediate direct cell death through a lysosomal pathway and engage ADCC but do not promote CDC or CD20 modulation.
entering the era of second- and third-generation anti-CD20 mAbs, which have augmented effector functions, such as higher affinity and enhanced FcR-binding. It will be very interesting to see how these modifications impact on the hallmark type I and II effector functions such as CDC, direct cell death, and CD20 modulation. In this regard, ofatumumab, which was recently approved by the US Food and Drug Administration for treatment of fludarabine- and alemtuzumab-refractory CLL (http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm188221.htm), probably reflects the gold standard type I mAb with its high affinity and powerful CDC activity, gained through its unique binding site, whereas GA101 (only the second type II mAb to translate into the clinic) represents an optimized type II, with good cell-killing activity and a glyco-engineered Fc for optimal ADCC. Therefore, it will be intriguing to observe how these clinical candidates perform over the next few years, perhaps allowing us to see if different types of CD20 mAbs are more suitable for particular types of disease.

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