Chapter 2
Resistance to Monoclonal Antibody Therapeutics in Lymphoma

Matthew J. Barth and Stanton C. Goldman

Abstract With the long history of rituximab use in CD20 positive lymphomas and the recent approval of brentuximab vedotin for the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma, monoclonal antibody-based therapies are commonly utilized for the treatment of many lymphomas. Following decades of experience with rituximab, much has been learned about the mechanisms of action and potential mechanisms of resistance to monoclonal antibody therapies, but a thorough understanding of which mechanisms of action are most relevant to rituximab's efficacy and which resistance mechanisms are most clinically relevant is still elusive. Nonetheless, many approaches have been identified and continue to be investigated both pre-clinically and clinically to attempt to overcome or circumvent resistance to monoclonal antibody therapies in order to enhance treatment responses or improve survival at the time of relapse following monoclonal antibody based therapy.

Keywords Monoclonal antibody · Antibody drug conjugate · Non-Hodgkin lymphoma · Hodgkin lymphoma · Resistance

M. J. Barth (✉)
Division of Pediatric Hematology/Oncology, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, NY, USA

Department of Pediatrics, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA
e-mail: Matthew.Barth@RoswellPark.org

S. C. Goldman
Division of Pediatric Hematology/Oncology, Medical City Children's Hospital, Dallas, TX, USA
e-mail: Stan.Goldman@usoncology.com

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Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
ADPC	Antibody-Dependent Phagocytic Cytotoxicity
AKT	Protein Kinase B
ALCL	Anaplastic Large Cell Lymphoma
ALL	Acute Lymphoblastic Leukemia
B-NHL	B-cell Non-Hodgkin Lymphoma
BiTE	Bispecific T-cell Engaging
CDC	Complement Dependent Cytotoxicity
CLL	Chronic Lymphocytic Leukemia
DLBCL	Diffuse Large B-Cell Lymphoma
EFS	Event-Free Survival
ERK1/2	Extracellular signal Related Kinase 1 and 2
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HACA	Human Anti-Chimera Antibodies
IFN-γ	Interferon Gamma
IL-2	Interleukin 2
IL-4	Interleukin 4
MAPK	Mitogen Activated Protein Kinase
MMAE	Monomethyl Aurostatin E
MS4A1	Membrane Spanning 4-Domain A1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NK-κB	Nuclear Factor Kappa B
NK-cell	Natural Killer Cells
PCD	Programmed Cell Death
PLCγ2	Phospholipase C Gamma 2
RIC	Radioimmunoconjugate
ROS	Reactive Oxygen Species
STAT3	Signal Transducer and Activator of Transcription 3
SYK	Spleen Associated Tyrosine Kinase
TNF-α	Tumor Necrosis Factor alpha

Introduction

The addition of monoclonal antibody therapy to the treatment of lymphoma has revolutionized its therapy over the past 2 decades. The proof of principle of monoclonal antibody therapies has been the addition of the anti-CD20 monoclonal antibody, rituximab, to therapy regimens for CD20 expressing mature B-cell lymphomas. The introduction of rituximab to the best backbone chemotherapy regimens for B-cell non-Hodgkin lymphoma (B-NHL) has improved event free survival (EFS) in high grade B-NHL. Well conducted randomized phase 3 studies have shown an approximately 15–20% absolute improvement in EFS (*vs*. chemotherapy alone) in

favor of rituximab in elderly patients with diffuse large B-cell lymphoma (DLBCL), younger patients with DLBCL and more recently adults with Burkitt lymphoma [1–3]. Until recently it was unknown whether the same would be true for pediatric mature B-NHL where the multiagent chemotherapy results alone were already greater than 80% survival. A recent international study in advanced pediatric Burkitt and DLBCL was halted early after the rituximab arm demonstrated a superior 1-year EFS (94%) compared to identical chemotherapy backbone alone (81%) [4]. Thus, rituximab (+ disease specific chemotherapy) is now considered standard of care in pediatric and adult patients with aggressive mature B-NHL.

While the success of rituximab is well documented, resistance to monoclonal antibody therapy has also been well described with multiple possible mechanisms of resistance reported. Numerous next generation monoclonal antibodies have been developed in an attempt to improve upon rituximab and circumvent mechanisms of resistance with varying degrees of success. Additionally, monoclonal antibodies modified to enhance interaction with host immune cells or conjugated to toxins or radiotherapeutic agents have been developed as an alternative approach to the use of naked monoclonal antibody therapies in the treatment of lymphoma. In this chapter, we will highlight resistance to monoclonal antibody therapies, focusing primarily on rituximab as the predominant monoclonal antibody utilized in the treatment of lymphoma, and the development of alternative approaches to overcome described mechanisms of resistance.

Resistance to Monoclonal Antibody Therapy in the Clinic

The efficacy of rituximab in treating B-NHL was first established in the setting of relapsed low-grade B-NHL where 4 weekly doses of rituximab single agent therapy led to responses in approximately 50% of patients in initial trials [5–8]. In patients with relapsed or refractory aggressive B-NHL variants, 8 weekly doses of rituximab led to responses in about 30% of patients [9]. In the setting of aggressive disease, patients with primary refractory disease, non-large cell variants and more bulky disease tended to be less likely to respond to single agent rituximab [9]. In low grade lymphoma patients having previously responded to rituximab, responses were noted in 40% of patients upon retreatment with single agent rituximab [10, 11]. These initial trials highlighted a failure to respond in more than half of relapsed patients treated with rituximab upon initial single agent treatment with more than half of initial responders developing resistance upon re-treatment. As an initial therapy for low grade B-NHL, rituximab induced a slightly higher response rate of greater than 60% as a single agent [12]. Rituximab also demonstrated the ability to sensitize lymphoma cells to the effects of cytotoxic chemotherapy and thus was subsequently combined with chemotherapy for treating both newly diagnosed and relapsed/refractory lymphoma patients. The combination of chemotherapy and immunotherapy with rituximab was initially investigated in the R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) with 95% of patients

with low grade B-NHL achieving a response [13, 14]. In the setting of aggressive B-NHL, similar response rates were noted [15]. Rituximab has subsequently been combined with a variety of chemotherapy regimens in both indolent and aggressive B-NHL and has become standard of care in the treatment of CD20-positive B-NHL. However, with the introduction of rituximab to front-line therapy for B-NHL, a new phenomenon of resistance has been noted in the relapse setting. A large Phase 3 study of relapsed DLBCL, the CORAL study, highlighted the development of resistance following treatment with rituximab containing regimens with patients having previously been treated with rituximab exhibiting an inferior survival upon treatment with rituximab containing salvage therapy compared to patients not having previously received a rituximab containing regimen [16]. The degree of contribution of rituximab to resistance is difficult to assess since current treatment essentially universally combines rituximab with chemotherapy. However, resistance has been noted both upon initial exposure and upon re-exposure to rituximab heightening interest in the mechanisms of resistance to monoclonal antibody therapies and the development of new immunotherapeutic agents able to overcome resistance.

Antibodies to Rituximab are Unlikely to Play a Role in Resistance

Monoclonal antibodies are large antigenic proteins and can theoretically be ineffective because of the formation of anti-antibodies, especially with repeated exposure. In addition, the less fully humanized antibodies are at higher risk of inducing an antibody response from the host. On the other hand, rituximab is a powerful humoral immunosuppressant with prolonged reduction of mature non-malignant B-cells and serum immunoglobulins. During our studies of the first trial of rituximab plus aggressive multi-agent chemotherapy in children and adolescents with *de novo* mature B-NHL, we could not demonstrate any formation of human anti-rituximab (HACA) antibodies [17]. In addition, by using a dose dense approach, we were able to demonstrate very high serum rituximab levels with t ½ of 26–29 days. Thus, reduced serum levels of antibody, through anti-antibody formation (or other mechanisms), is unlikely to play a role in resistance.

Mechanisms of Monoclonal Antibody Activity

To understand the mechanisms of resistance to monoclonal antibody therapy, one needs to initially understand the varying potential mechanisms of activity of monoclonal antibodies. Monoclonal antibodies can function to kill tumor cells through a variety of mechanisms. These primarily include antibody-dependent cellular

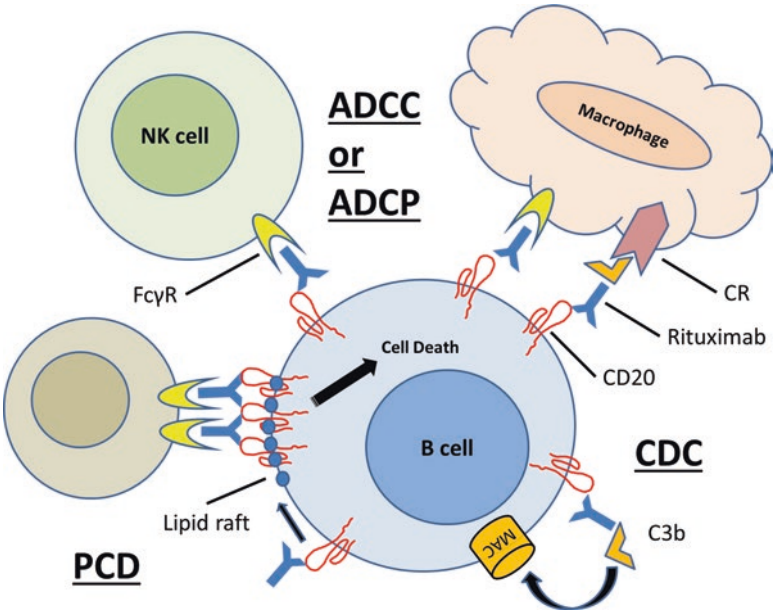

Fig. 2.1 Mechanisms of rituximab activity. Rituximab binding to surface CD20 leads to lymphoma cell death through several reported mechanisms. (1) Binding of rituximab leads to the activation of complement leading to the formation of the membrane attack complex (MAC) resulting in cell lysis. (2) Binding of the Fc portion of rituximab by Fcγ receptors (FcγR) or rituximab bound complement C3b by complement receptors (CR) on effector cells leads to cell killing by antibody dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP). (3) Binding of rituximab to CD20 leads to mobilization to lipid raft domains where crosslinking of CD20 bound rituximab leads to intracellular signaling and induction of apoptosis

cytotoxicity (ADCC) or phagocytic cytotoxicity (ADCP), complement dependent cytotoxicity (CDC) and direct induction of programmed cell death (PCD) (Fig. 2.1) [18–20]. Additionally, monoclonal antibodies can function to sensitize tumor cells to the effect of cytotoxic chemotherapy exhibiting synergistic activity in combination immunochemotherapy regimens [21]. The most relevant mechanism of action of an individual anti-CD20 antibody can largely be defined by whether the antibody is a type I antibody (e.g. rituximab) or a type II antibody (e.g. tositumomab, obinutuzumab). Type I anti-CD20 antibodies can localize CD20 into membrane lipid raft domains effectively activating complement and altering signal transduction through co-localization of receptors and effectors; while type II antibodies do not induce lipid raft localization and generally induce limited CDC, but more robust induction of PCD [22, 23].

ADCC/ADCP relies on the binding of the Fc fragment of the monoclonal antibody to receptors on surrounding immune effector cells [natural killer (NK) cells, monocyte/macrophages and neutrophils] inducing tumor cell death through triggering the immune effector cells to bind and kill the tumor. The role of ADCC on rituximab *in vivo* activity has been demonstrated by impaired activity in NK-cell

and neutrophil depleted mice and Fc receptor dependent activity [24, 25]. CDC relies on activation of the complement cascade through binding of the protein C1q to the Fc portion of the antibody leading to development of a membrane attack complex leading to cell lysis. The dependence on complement for activity has been demonstrated by a lack of rituximab activity in mice deficient in C1q or with complement depleted by exposure to cobra venom factor, though others have also demonstrated that deficiency of complement proteins had little impact on rituximab activity suggesting that Fc-receptor dependent ADCC activity was more critical to rituximab activity *in vivo* [25, 26]. Additionally, some have suggested that complement activation may impair other antibody mediated mechanisms of cell killing like ADCC [27]. This detrimental effect of complement activation has also been suggested in relation to an increase in progression free survival noted in follicular lymphoma and DLBCL patients with mutations in the gene encoding C1q that are known to cause lower C1q expression [28, 29].

While the direct induction of PCD by monoclonal antibodies has been demonstrated *in vitro*, the mechanism of such an effect *in vivo* has been difficult to demonstrate so that less is understood about the exact mechanism of antibody induction of cell death. The mechanism of induction of cell death also likely varies between antibody types with type I and type II anti-CD20 monoclonal antibodies demonstrating varying mechanisms. Rituximab binding to CD20 on the surface of malignant B-cells has been shown to induce a caspase-dependent apoptosis through activation of caspases 3 and 9 leading to PARP cleavage with these effects being inhibited by exposure to caspase inhibitors and enhanced by cross linking of CD20 bound rituximab molecules [30–32]. While this suggests a caspase-dependent mechanism of cell death induction, others have reported cell death associated with rituximab binding that is independent of caspase activation and resistant to caspase inhibition [33]. Apoptosis induction may also be dependent on altered calcium transport leading to increased intracellular calcium following rituximab exposure with calcium chelators inhibiting the apoptosis induced by rituximab [31, 32]. The cellular function of membrane bound CD20 is likely to be a calcium channel critical to B-cell signaling. This shift in intracellular calcium after rituximab binding to CD20 has been shown to be secondary to activation of Src-family protein tyrosine kinases leading to phosphorylation of phospholipase C gamma 2 (PLCγ2) [34]. Additional intracellular signaling effects reported following rituximab binding have been noted on the mitogen activated protein kinase (MAPK), extracellular signal related kinase 1 and 2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) signaling pathways [35–38]. Rituximab binding has also been shown to alter expression of Bcl-2 family member proteins and other inhibitors of apoptosis proteins. Chemosensitization observed following rituximab exposure may in large part be due to the documented ability of rituximab to overcome Bcl-2 associated resistance following chemotherapy exposure [30, 39]. Some have also theorized that debris from apoptotic cells can have a "vaccination effect" leading to expansion of lymphoma specific cytotoxic T lymphocytes [40]. This effect has been demonstrated in mice where tumor re-challenge in mice previously treated with an anti-CD20 antibody led to impaired engraftment

Table 2.1 Mechanisms of resistance to monoclonal antibody therapy and approaches to overcoming resistance

Mechanisms of activity	Mechanisms of resistance	Approaches to circumventing resistance
CDC	Complement depletion [49, 71, 72]	Next generation mAbs with enhanced CDC activity [82–84]
	Complement variants [26]	Enhanced hexamer foundation [99]
	Complement inhibitory proteins [73–79]	Complement replacement [71, 72]
ADCC	FcγR polymorphisms [102, 103, 105, 108, 110, 111, 113, 118]	Next generation mAbs with enhanced FcR affinity [121–129]
	Inhibitory FcγR expression [100, 119, 120]	
PCD/ Apoptosis	Altered Bcl-2 protein expression/ intracellular signaling [146, 147]	Type II antibodies with enhanced cell death induction [21, 124, 128, 150, 151]
Antigen binding	Antigen variants [46, 52, 53]	Increase CD20 expression using epigenetic modulating agents or cytokines [19, 59–64, 67, 68]
	Antigen shedding [48, 49]	Type II monoclonal antibodies [51]
	Antigen internalization [50]	Dose dense dosing [17, 143–145]
	Circulating antigen [138–142]	
ADC	Drug transporter mediated efflux [158]	Alternative anti-neoplastic conjugates [160]

CDC, complement dependent cytotoxicity; ADCC, antibody dependent cellular cytotoxicity; PCD, programmed cell death; ADC, antibody-drug conjugate; mAB, monoclonal antibody

and the identification of macrophage-associated ADCC leading to dendritic cell uptake of immune complexes inducing anti-tumor adaptive responses [41–43].

The exact role and contribution of each mechanism of activity to the efficacy of monoclonal antibody therapies is still not clearly understood and is likely disease- and antibody-dependent. Each of these mechanisms of activity has also been associated with proposed mechanisms of resistance (Table 2.1).

Mechanisms of Monoclonal Antibody Resistance

Antigenic Alterations Leading to Resistance

For a monoclonal antibody to exert its effect, it needs to first bind to its target antigen. The level of expression or mutations in the surface antigen to which an antibody is targeted can impact the activity of the monoclonal antibody. One of the characteristics of CD20 that was believed to make it an ideal antigen for antibody targeting was a reported lack of internalization or shedding of the protein [18]. Despite this, early in the investigation of rituximab, reports began emerging

describing the loss of CD20 expression in patients with relapsed B-NHL following exposure to anti-CD20 monoclonal antibody therapy [44–47]. Though the relative incidence of CD20 loss after rituximab exposure in the clinic has generally been believed to be low, investigation of alterations of CD20 expression levels in rituximab resistant cells has indicated a possible role of this phenomenon in rituximab resistance.

In rituximab-resistant B-NHL cell lines developed by serial exposure of cell lines in culture to rituximab, decreased CD20 expression has been described in the resultant resistant cells reported to be due to transcriptional and post-transcriptional mechanisms [48]. Alternative splicing of CD20 mRNA may also impact rituximab response with an alternatively spliced, truncated version of the CD20 protein reported in B-lymphocytes that were either malignant or EBV transformed, but not present in non-transformed B-cells [49]. The variant CD20 was noted to increase in expression in rituximab-resistant cell lines developed by exposure to rituximab in vitro and also in primary patient cells following exposure to rituximab suggesting a role in development of rituximab resistance.

CD20 expression may also be altered after rituximab exposure secondary to antigenic modulation or "shaving". Beum *et al*. described a so called "shaving effect" leading to loss of CD20 expression on malignant B-cells [50]. The described effect was reported both clinically, with reported rapid loss of CD20 and rituximab from B-cells without internalization, and in an experimental system where rituximab-CD20 complexes were noted to be removed from B-cells and taken up by monocytes in co-culture [51, 50]. Some reports have also suggested that, contrary to earlier data, CD20 may be internalized following rituximab binding. Beers *et al*. demonstrated using fluorescently labeled rituximab that internalization of the rituximab-CD20 complex occurred following rituximab exposure with trafficking of rituximab-CD20 complexes noted to endosomes and lysosomes in B-cells [52]. Variability in the internalization of CD20 was noted with different types of monoclonal antibodies with the Type I rituximab antibody leading to internalization while a Type II tositumumab-like antibody did not, highlighting potential differences in the mechanism of action and resistance to different antibody constructs [52]. Utilization of a Type II antibody, like the humanized, glycoengineered Type II anti-CD20 monoclonal antibody obinutuzumab may thus allow for activity without significant modulation from internalization, though recent studies have also reported on the "shaving phenomenon" occurring with obinutuzumab as an alternate mechanism for resistance in the absence of antigenic modulation [53].

In addition to antibody associated effects leading to altered expression of the CD20 antigen on the B-cell surface, others have reported on mutations in the gene encoding CD20 that may impair response to rituximab [54, 55]. For example, Turui *et al*. performed a mutation analysis of CD20 in 50 patients treated for a variety of NHL types including 9 patients with progressive disease [55]. They found that 11 patients (22%) had a mutation in CD20 and that those with a C-terminal deletion mutation had a significantly lower expression of CD20 compared to patients without a mutation or those with mutations defined as early termination or extracellular domain. Notably 4 of the 5 C-terminal deletions occurred in samples from patients

with progressive disease. Cells transfected with the C-terminal deletion mutated CD20 expressed similar CD20 RNA, but did not express CD20 on the cell surface with only weak cytoplasmic staining noted. These C-terminal mutations were subsequently reported to affect the extracellular large loop of the CD20 antigen also impacting the rituximab binding site [56]. Another relapse case was noted to have a homozygous deletion of the membrane spanning 4-domains A1 (MS4A1) gene, the gene encoding CD20, at relapse leading to loss of CD20 [57]. While these mutations have been reported, a larger analysis of DLBCL patients identified that such mutations occur at very low rates (0.4% of 264 newly diagnosed and 6% of 15 relapsed DLBCL patients analyzed) and may not significantly contribute to resistance except in a small percentage of cases [58].

Epigenetic regulation of the gene encoding CD20 has also been implicated in changes in CD20 surface antigen expression and thus possibly also related to rituximab resistance [59, 60]. Tomia *et al.* reported on a case of CD20 negative relapsed DLBCL after rituximab exposure with increased CD20 expression following exposure to the epigenetic modifier Trichostatin A [59]. A further analysis of mechanisms of epigenetic regulation of decreased CD20 expression identified the role of the Sin3A-HDAC1 co-repressor complex in downregulating transcription of MS4A1 with expression of CD20 increased following exposure to the histone deacetylase inhibitor Trichostatin A [60]. Similarly, numerous epigenetic modifying agents have been identified that can alter CD20 protein expression and augment the activity of rituximab through their effects on DNA methylation, DNA acetylation or the recruitment of transcription factors leading to altered CD20 expression [61–66]. The potential clinical impact of epigenetic modifiers has also been investigated in combination with rituximab containing regimens with some promising early findings [67, 68]. Alternative mechanisms of increasing CD20 expression have been reported using a variety of cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-4 and IL-2 suggesting possible roles of combination therapies involving cytokine based therapies to increase CD20 expression prior to rituximab therapy [69, 70, 21]. An additional approach to enhance targeting of tumor cells using monoclonal antibodies is the use of multivalent antibodies targeting multiple antigens on a tumor cell or having multiple binding sites for a single antigen. Examples include an antiCD20/CD22 bivalent antibody which has demonstrated enhanced in vitro and in vivo cell killing compared to the individual antibodies or a combination of the two single antibodies [71]. A combination of a type 1 and a type 2 CD20 antibody into one bivalent antibody also exhibited enhanced CDC and direct killing [72]. In addition to approaches intended to enhance expression of CD20 to improve response to rituximab, antibodies targeting alternative lymphoma associated cell surface antigen targets continue to be developed for use in the setting of rituximab resistance or CD20 negative relapsed disease including, for example, monoclonal antibodies targeting CD19, CD22, CD79b, CD80 and CD40 with varying degrees of activity [73–78].

Complement Mediated Resistance

Binding of monoclonal antibodies to surface proteins can induce CDC via interactions of the Fc portion of the antibody with complement proteins. In chronic lymphocytic leukemia (CLL), a rapid depletion of complement proteins has been observed which may represent a limitation of rituximab activity [51]. This possible source of resistance was further supported by evidence that infusing rituximab with complement containing fresh frozen plasma may enhance rituximab activity [79, 80]. Polymorphisms in genes encoding C1q have also been reported to impact rituximab activity in patients with follicular lymphoma also highlighting the potential important role of CDC in rituximab activity, especially when given without chemotherapy [28].

Tumor cells can also inhibit CDC killing through the expression of complement inhibitory proteins CD46, CD55 and CD59 with altered expression of complement inhibitory proteins identified as a possible mechanism of resistance to monoclonal antibody therapies [81]. B-NHL cells resistant to rituximab, including tumor cell lines and primary patient cells, have been shown to exhibit increased expression of CD55 and CD59 leading to impaired CDC activity of anti-CD20 monoclonal antibodies [82–85]. The effect of complement inhibitory proteins on the CDC activity of rituximab has been demonstrated through increased rituximab associated CDC following inhibition of CD55 or CD59 [84, 81, 86, 87]. Despite this in vitro evidence of the detrimental effect of high complement inhibitory protein expression on rituximab activity, clinical investigation of the effect of high complement inhibitory protein expression on treatment response to rituximab has been conflicting with some analysis suggesting higher levels of CD55 and CD59 in non-responders while others suggest no impact of varying levels of CD46, CD55 or CD59 on likelihood of response to rituximab [88, 89].

Novel monoclonal antibodies have been developed which exhibit enhanced CDC activity in comparison to rituximab [90]. CDC activity has been linked to the proximity of antibody binding to the cell membrane and is dependent on redistribution of the antigen target into lipid soluble rafts within the cell membrane, an effect predominantly observed with Type I antibodies [91, 90]. The fully human type I anti-CD20 monoclonal antibody ofatumumab binds to a unique, more membrane-proximal epitope of the CD20 antigen compared to rituximab and has a slower off-rate while effectively inducing CD20 redistribution to lipid rafts [90, 92]. Likely secondary to these characteristics, ofatumumab has demonstrated enhanced CDC activity in comparison to rituximab including in the setting of rituximab resistance and high levels of CD55 and CD59 expression [90, 83, 93–95]. Clinically, ofatumumab has induced a high rate of responses, particularly in CLL alone or in combination with chemotherapy, and has received FDA approval for first line and refractory CLL alone and with various alkylator combinations. [96–100]. Ofatumumab demonstrated limited efficacy in aggressive B-NHL, where no significant benefit was observed over rituximab, though some responses have been noted

in rituximab resistant disease. This highlights that CDC may play a larger role in certain B-cell malignancies (like CLL) compared to others [101–105].

An alternative approach to enhancing monoclonal antibody associated CDC relates to the formation of antibody hexamers in order to activate complement effectively. This recently described hexamer formation of anti-CD20 antibodies increases C1q binding and enhances CDC activity [106]. Polymorphisms in the Fc portion of an antibody have been identified that enhance hexamer formation and thus increase CDC activity of the antibody. Introducing such polymorphisms into rituximab was shown to increase CDC in CLL samples and was also even shown to increase CDC induced by type II anti-CD20 monoclonal antibodies [107]. This represents another potential approach to overcoming resistance to CDC activity.

Fcγ Receptor Associated Resistance

Much of the function of rituximab and other monoclonal antibodies is dependent on the interaction of the Fc portion of the antibody with Fcγ receptors (FcγR), in particular FcγRIIIa and FcγRIIa receptors on myeloid effector cells [108, 25, 109]. FcγR deficiency in mice abrogates the activity of monoclonal antibody therapies providing evidence for their crucial role in monoclonal antibody activity [108]. Polymorphisms in FcγR leading to altered affinity for Fc binding have been noted to impact the efficacy of rituximab in vitro and in vivo. In particular, the 158F variant of FcγRIIIa has been noted to impair responsiveness to rituximab compared to the 158V variant which has a higher affinity for binding IgG1 antibodies [110]. In patients with previously untreated follicular lymphoma treated with rituximab, response rates were significantly higher in patients homozygous for the FcγRIIIa 158V variant compared to 158F carriers [111]. However, subsequent analysis in a variety of tumor types have provided conflicting results in particular in patients treated with rituximab in combination with chemotherapy [112–122]. There may also be an effect on toxicity associated with rituximab exposure as the high affinity FcγRIIIa 158V polymorphism has recently been associated with increased rates of late onset neutropenia following rituximab therapy [123–125]. Polymorphisms in FcγRIIa have also been implicated in response to rituximab, in particular the FcγRIIa H131R polymorphism which has been associated with improved response in tumors with a higher affinity H/H genotype, though similar to the FcγRIIIa polymorphisms, the impact on clinical outcome has been mixed with no impact noted in most recent studies of rituximab in combination with chemotherapy [121, 119, 118, 126, 116, 113].

Additionally, the expression of other inhibitory FcγR, such as FcγRIIb, may impair response upon binding of effector macrophages [108]. This has been demonstrated in transgenic mice lacking the FcγRII inhibitory receptor, in which tumors tend to be more responsive to monoclonal antibody therapies [108]. FcγRIIb has also been reported to interact with rituximab bound to CD20 to form a complex that promotes internalization of the rituximab-CD20 complex impairing Fc-dependent

functions and overall antibody efficacy [127, 52]. The clinical effect of high FcγRIIb has also been described in follicular lymphoma patients receiving rituximab monotherapy where patients with high FcγRIIb expression exhibited lower EFS [128].

With variability in FcγR binding affinity playing a potential role in response to rituximab, novel monoclonal antibodies have been developed with alterations aimed at enhancing Fc receptor affinity. Alterations to the Fc portion of monoclonal antibodies have improved affinity for lower affinity FcγRs leading to improved ADCC activity. Afucosylation (manipulation of the oligosaccharides to remove fucose) of the Fc portion of antibodies was shown to decrease steric hindrance that likely inhibited FcγR binding leading to enhanced receptor affinity and increased ADCC [129–131].

Obinutuzumab is the prime example of a third generation type II, humanized, glycoengineered anti-CD20 monoclonal antibody. Obinutuzumab has been shown to exhibit enhanced pre-clinical activity compared to rituximab both from enhanced ADCC and from enhanced direct cell killing typical of type II antibodies [132–137]. Despite this promise related to pre-clinical activity, the clinical development of obinutuzumab has led to variable results. In CLL, as a single agent, obinutuzumab induced a rapid decrease in circulating CD20 positive cells associated with a significant rate of infusion related reactions secondary to cytokine release [138]. In combination with chlorambucil in newly diagnosed CLL patients with coexisting conditions, obinutuzumab demonstrated high response rates and progression free survival compared to chlorambucil monotherapy or the combination with rituximab [139]. In patients with relapsed/refractory indolent NHL, obinutuzumab monotherapy induced responses in 62% of patients during a phase 1 dose escalation and 30% in the phase 2 portion of the trial including responses in patients having previously received rituximab [140]. The best overall response rate of all patients also seemed to be higher than that reported with ofatumumab as monotherapy in a similar population. A subsequent randomized study of obinutuzumab in comparison with rituximab reported a similar response rate of 44.6% in obinutuzumab treated patients compared to 26.7% with rituximab, as determined by a blinded review panel [138]. However, despite the apparent benefit in response, obinutuzumab did not lead to an improvement in progression free survival [138]. Due to an observed dose response effect with obinutuzumab, a randomized trial of 1000mg vs 2000mg was performed which seemed to confirm a higher response rate with increased dosing (67% vs 49%) in previously untreated CLL patients [141]. In patients with relapsed indolent NHL following prior rituximab containing therapy, obinutuzumab was randomly studied in combination with bendamustine compared to bendamustine alone with obinutuzumab maintenance given in patients responding to the combination. While the end of induction response rate was no different between the two arms, the obinutuzumab/bendamustine group experienced less events and had a prolonged progression free survival compared to bendamustine alone [142]. In DLBCL, obinutuzumab monotherapy resulted in responses in 32% of patients, a rate that is similar to responses to rituximab in rituximab naïve relapsed DLBCL patients, with 20% of 25 rituximab-refractory patients achieving a response and a suggestion of increased

responses in a higher dose group [143]. In newly diagnosed DLBCL patients, obinutuzumab/CHOP was compared to rituximab/CHOP with no difference in EFS noted [144]. Obinutuzumab has gained regulatory approval in the United States for treatment of newly diagnosed CLL in combination with chlorambucil and in combination with bendamustine followed by obinutuzumab monotherapy in patients with follicular lymphoma relapsed after a rituximab containing regimen. However, the results in aggressive NHL variants have been inconsistent and continue to be evaluated with no current indication for aggressive B-NHL to date.

Circulating Antigen

Many cell surface antigens can also be identified in circulation. These circulating CD20 (cCD20) antigens have been identified in patients with CLL, Hodgkin lymphoma and NHL in addition to healthy controls [145–147]. Patients with B-NHL had significantly higher levels of cCD20 compared to normal controls [146]. In CLL, high levels of cCD20 have been correlated with disease stage and inversely correlated with overall survival [148]. It has been suggested that high levels of cCD20 may complex with therapeutic monoclonal antibodies leading to enhanced clearance, a mechanism suggested to contribute to the impaired efficacy of rituximab in CLL [149]. A more recent report also suggested a role of cCD20 in clinical outcomes in B-NHL with patients with high cCD20 levels prior to receiving therapy and those with higher cCD20 after therapy having a significantly lower probability of survival [146]. Serum rituximab concentration has been correlated to response in some studies with patients achieving higher concentrations being more likely to respond and patients with higher disease burden generally attaining less ideal rituximab levels [150–152, 17]. Binding of rituximab to cCD20 may hinder it's binding to B-cell associated CD20 and possibly increase clearance leading to decreased rituximab concentrations which may be able to be overcome by increased rituximab dose intensity.

Resistance to Apoptosis

As previously discussed, binding of monoclonal antibodies to surface antigens can induce intracellular signals leading to induction of apoptosis without the need of third party effector cells or complement activation. Alterations in the signaling pathways leading to apoptosis can thus lead to impaired ability of antibody to induce this effect. Multiple groups have generated NHL cell lines resistant to rituximab following serial exposure to the antibody and have demonstrated that alterations in pro- and anti-apoptotic regulators of apoptosis likely contribute to the development of resistance [153, 154]. While rituximab has demonstrated the ability to induce apoptosis, the importance of this effect on lymphoma cell death is unclear.

Type II anti-CD20 monoclonal antibodies on the other hand have demonstrated a more significant induction of cell death when compared to rituximab. In experiments assessing the cell killing effect of Type I *vs.* Type II antibodies, $F(ab')_2$ fragments of Type II antibodies were able to induce significant cell death independent of Fc dependent mechanisms as opposed to the Type I antibody which required the Fc fragment to induce cell death primarily through complement activation [23]. Type II antibodies have been developed in order to improve on the cell death induction observed with rituximab. Obinutuzumab has also demonstrated significantly more induction of cell death than rituximab *in vitro* [132]. While as previously discussed, obinutuzumab has enhanced ADCC activity secondary to a glycoengineered Fc segment increasing FcR binding affinity, the same antibody without the glycoengineering still maintained superior cell killing compared to rituximab highlighting the increased induction of PCD by this type II antibody [155]. Obinutuzumab, similar to a previously developed Type II antibody tositumomab, induces a caspase independent cell death that correlates with high levels of homotypic adhesion not observed with rituximab, possibly indicating enhanced signaling effect [22, 133]. This caspase-independent cell death has also been identified to occur through the generation of reactive oxygen species (ROS) mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase independent of mitochondria and can be blocked by exposure to an ROS scavenger [156]. Though additional signaling effects of obinutuzumab compared to rituximab that may be related to an increased induction of cell death and possible chemosensitization continue to be investigated, there do appear to be differences in signaling effects compared to rituximab in cells that are either rituximab-sensitive or rituximab-resistant with more significant effects noted on activation of protein kinase B (AKT), spleen-associated tyrosine kinase (SYK) and PLCγ2 following obinutuzumab exposure *in vitro* [157, 158].

Alternative Antibody Mediated Therapeutics

In addition to the use of naked monoclonal antibodies, other immunotherapy approaches utilize antibody-based approaches to direct therapy. Antibody drug conjugates (ADCs) or radioimmunoconjugates (RIC) utilize an antigen targeting antibody to deliver a drug or radioactive molecule that is covalently bound to the antibody to tumor cells in a targeted fashion. RICs have been thoroughly investigated in B-NHL and have earned regulatory approval for some limited treatment indications including indolent lymphomas relapsed after rituximab therapy (I^{131}-tositumomab and ibritumomab tiuxetan) or newly diagnosed follicular lymphoma following a response to initial therapy (ibritumomab tiuxetan).

In addition to these radioimmunoconjugates, other antibody drug conjugates have been evaluated in lymphoma. The most established is Brentuximab vedotin, an ADC targeting CD30 conjugated with monomethyl auristatin E (MMAE), a potent microtubule stabilizing agent. Brentuximab vedotin demonstrated significant

responses in relapsed refractory Hodgkin lymphoma and anaplastic large cell lymphoma, two lymphoma types with high CD30 expression; and it is approved for use in adult classical Hodgkin lymphoma patients who have relapsed after stem cell transplant or 2 chemotherapy regimens, as consolidation after transplant or with newly diagnosed stage 3 or 4 disease and in relapsed systemic or anaplastic large cell lymphoma or mucosis fungoides [159, 160]. Despite low levels of CD30 expression, brentuximab vedotin has also exhibited activity in treatment of some B-NHLs, in particular DLBCL and primary mediastinal large cell lymphoma [161].

Since these agents rely on the antibody primarily for targeting purposes, resistance mechanisms relating to Fc associated mechanisms of activity previously discussed are generally less relevant. However, changes in antigen expression can have a role in resistance to ADCs which continue to rely on antigen expression for appropriate delivery of their cargo. A single case of CD20 negative relapse following treatment with I^{131}-tositumomab has been reported though this was a very early progression raising the question of monoclonal antibody blocking binding of anti-CD20 antibody used for immunohistochemistry analysis [162]. Additionally, CD30 negative relapse of ALCL has been reported following treatment with brentuximab vedotin [163, 164].

Similarly, since the efficacy of ADCs is dependent on the anti-neoplastic agent conjugated to the antibody, additional mechanisms of resistance common to other chemotherapeutic agents can contribute to resistance. Chen *et al.* described mechanisms of resistance to brentuximab vedotin in ALCL and Hodgkin lymphoma cell lines generated to be resistant following serial exposure [165]. In addition to down-regulation of CD30 expression affecting ADC targeting, resistance to MMAE was observed. MMAE intracellular accumulation was lower in resistant cells following exposure to the ADC or to free MMAE suggesting possible impaired delivery related to decreased antigen expression, but also resistance to MMAE itself. Investigation of mechanisms of resistance to the MMAE identified an increased expression of the multi-drug resistance gene MDR1, with resistance to MMAE partially reversed following inhibition of p-glycoproteins. Similar increase in positivity for drug transporters was observed in patient samples from relapses following brentuximab vedotin therapy.

Additionally, altered induction of target cell apoptosis may contribute to resistance to the conjugated molecule. For example, in pre-clinical investigation of a novel ADC targeting CD79b and conjugated with MMAE, increased expression of the anti-apoptotic Bcl-2 family protein Bcl-xL was demonstrated to be associated with resistance to this investigational ADC with enhanced responses noted following inhibition of Bcl-2 family proteins using ABT-263 [166]. Alternative antibodies with enhanced antigen targeting or targeting alternative surface antigens, conjugates with more efficient conjugation of anti-neoplastic compounds and alternative anti-neoplastic agents not known to be substrates for drug transporter mediated efflux represent potential options for alternative ADCs to circumvent these identified mechanisms of resistance.

Another use of monoclonal antibody therapy is the ability to target tumor cells to cytotoxic effector cells using bi-specific antibodies that can bind the target cell and an effector cell. The prime example is the bispecific T-cell engaging (BiTE) antibody blinatumomab. Blinatumomab is a bivalent antibody targeting CD19 present on B-cells and CD3 on T-cells leading to enhanced immune-mediated clearance of tumor cells. It has been approved for use in acute lymphoblastic leukemia (ALL) and is under investigation in B-NHL with responses in 69% of 76 relapsed/refractory B-NHL patients including 55% of patients with DLBCL [75]. With data primarily in ALL, resistance to blinatumomab has been noted with CD19-negative relapses and primary resistance possibly due to high expression of the checkpoint inhibitor ligand PD-L1 on tumor cells [167–169]. Alternative bispecific antibodies constructed to enhance immune surveillance of malignant cells continue to be developed and evaluated in B-NHL including a CD20-CD3 bispecific antibody [170].

Summary

Monoclonal antibodies have been a cornerstone of therapy for lymphomas for decades following the first ever approval of a monoclonal antibody therapy, rituximab, for the treatment of cancer. Despite the overwhelming success of rituximab in treating NHLs, resistance exists both in primary refractory cases and on relapse following treatment with rituximab. Mechanisms of resistance have been identified that target all described mechanisms of monoclonal antibody activity including altered antigen expression or binding, impaired CDC or ADCC, altered intracellular signaling effects and inhibition of direct induction of cell death. Multiple next generation anti-CD20 monoclonal antibody therapies developed to overcome described resistance mechanisms continue to be investigated with two, ofatumumab and obinutuzumab, already approved for the treatment of B-cell malignancies albeit with narrow indications. Alternative monoclonal antibody based immunotherapeutic approaches more recently developed include the use of ADCs and bispecific or multivalent antibody constructs. The ADC brentuximab vedotin has approvals for indications in Hodgkin lymphoma and relapsed ALCL, while the BiTE antibody blinatumomab is approved for use in B-ALL. Understanding of these newer monoclonal antibody based therapeutic approaches and mechanisms of resistance to them continue to be studied, with alternative agents from each class already in development to try to improve on the significant activity already observed with each agent.

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