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Rituximab: mechanism of action

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Abstract

Rituximab is a mainstay in the therapy for a broad variety of B-cell malignancies. Despite its undeniable therapeutic value, we still do not fully understand the mechanisms of action responsible for rituximab's anti-tumor effects. Direct signaling, complement dependent cellular cytotoxicity and antibody dependent cellular cytotoxicity all appear to play a role in rituximab efficacy. In vitro, animal model and clinical data addressing each of these mechanisms of action are reviewed, as are data speaking to the complexity of interactions between these mechanisms. Taken together, these data suggest different mechanisms are likely important in different scenarios. Study of the complex mechanisms of action that contribute to the clinical efficacy of rituximab have led to novel clinical trials including novel combinations, schedules, and generation of additional antibodies designed to have even greater effect. Such studies need to be accompanied by rigorous correlative analysis if we are to understand the importance of various mechanisms of action of rituximab and use that information to improve on what is already an indispensable approach to therapy.

Overview – Interpreting results related to rituximab mechanisms of action

Rituximab has become a mainstay in the therapy of a broad variety of B-cell malignancies. In some B cell malignancies, rituximab alone can induce high response rates and long term remissions (1,2) while in others, adding rituximab to chemotherapy enhances the complete response, long term remission and cure rate (3,4). Despite its undeniable value as a component of therapy for anti-B cell malignancies, rituximab is not effective for all patients, and development of resistance to therapy is common. Understanding the mechanisms by which rituximab induces anti-tumor responses is central to our ability to improve on what is already a highly effective therapy.

We know that anti-cancer monoclonal antibodies (mAbs) can mediate anti-tumor effects by a variety of mechanisms including signaling resulting in cell cycle arrest, direct induction of apoptosis, and sensitization to cytotoxic drugs, complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC). Ideally, we would study these, and any other, mechanisms of action of rituximab using experimental conditions that reflect clinical therapy. Unfortunately, this is more easily said than done. As a single agent, rituximab is usually administered weekly for 4 weeks. When used in combination with chemotherapy, it is often administered every 3-4 weeks. The pharmacokinetics of rituximab is similar to that seen with human IgG (5). Thus, whether given weekly or monthly, rituximab is present at therapeutic levels in the circulation of patients for months at a time. As an IgG, rituximab distributes in both the intravascular and extravascular compartments, and so should be present within

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involved lymph nodes with their complex architecture in an environment that includes not only malignant B cells, but also stromal cells, benign lymphocytes, extracellular matrix, vasculature, proteins in the extravascular fluid, and a complex mixture of cytokines and chemokines.

In vitro studies allow for the rapid, rigorous and focused evaluation of specific mechanisms of action. However, the conditions we have available in the research laboratory vary significantly from the real-world clinical environment. Studies of rituximab mechanisms of action often utilize rapidly dividing tumor lines that have been selected based on their ability to grow rapidly in vitro, and sometimes their relative sensitivity to therapy. Effector cells, when present, are usually not syngeneic and often come from normal donors, not patients with malignancy. In vivo, lymphocyte behavior changes within seconds of cells being exposed to hypoxic conditions (6). It takes minutes to hours to harvest, wash and otherwise manipulate peripheral blood cells for in vitro analysis. Obtaining malignant lymphocytes from lymph nodes involves much more drastic manipulation, and often they are cryopreserved and then thawed before analysis. These manipulations surely have effects on their response to therapy. Our best in vitro assays involve incubation times of minutes (analysis of direct signaling effects of rituximab) to hours (cytotoxicity assays), but never weeks or months – the time frame of clinical response to rituximab. Finally, in vitro assays usually focus on one mechanism. Most studies of CMC do not include immune effector cells, and studies of ADCC do not have functional complement, thereby preventing these analyses from informing us about interactions between mechanisms.

Animal models come one step closer to reflecting the clinical situation but also have significant limitations. Such models traditionally involve mice that have been inoculated with malignant cell lines. Resulting tumors differ from clinical lymphoma with respect to growth kinetics, phenotype, infiltrating benign cells and heterogeneity. Many models utilize immune compromised animals as hosts, and xenografted human tumors. Furthermore, experimental conditions (tumor burden at time of therapy, dosing of therapy, etc) are usually selected with an eye towards enhancing our ability to detect a therapeutic response as opposed to understanding the mechanisms responsible for that therapeutic response. Each of these factors impacts on our ability to relate animal model results to clinical mechanisms of action.

Clinical trials and correlative assays are extremely valuable as they involve measurement of what is happening in patients. However, the vast majority of clinical trials are designed to assess efficacy of therapy, not understand mechanisms of action. This restricts our ability to manipulate conditions in a way that explores specific mechanisms. Correlative laboratory evaluation associated with clinical trials has proven to be extremely valuable, but even when informative, usually leads to a demonstration of a correlation rather than causation - they are more often hypothesis generating than hypothesis testing.

These limitations do not invalidate the significance of the information gained from such research but need to be taken into account when interpreting experimental results. This review will discuss what we know, and don't know, about the mechanisms of action of rituximab. It has been organized based on proposed mechanism of action, with a description of data available from in vitro, in vivo and clinical trials for each proposed mechanism. The primary mechanisms by which rituximab likely mediates its anti-tumor activity do not occur independently. The final sections of this review involves discussion of possible interactions between mechanisms, and how these interactions might impact on the efficacy, or resistance to, rituximab-based therapy.

1 - Signaling induced cell death

1A. In vitro studies

In the absence of immune effector mechanisms rituximab can induce death of malignant B cell lines in vitro. The strength of this effect varies considerably between target cell lines (7-10). Signaling mediated by cross-linking of CD20 appears to be related to functional reorganization of CD20 into lipid rafts. Changes that have been identified in response to rituximab in vitro include inhibition of p38 mitogen-activated protein kinase, NF- κ B, extracellular signal-regulated kinase 1/2 (ERK 1/2) and AKT antiapoptotic survival pathways. Development of in vitro resistance to rituximab is not associated with genetic changes in the CD20 molecule, but has been found to be associated with down-stream changes in signaling (11). The selection of CD20 resistant clones in these studies was done in the absence of immune effector mechanisms (complement or cells capable of mediating ADCC), thus whether these changes also result in clinical resistance to rituximab remains unclear.

Detection of signaling changes in response to rituximab often requires cross-linking of the rituximab with secondary antibodies. The affinity of anti-rituximab antibodies for rituximab is much higher than the affinity of rituximab Fc for FcR, and there is considerable cross-linking because of the bivalent nature of the cross-linking IgG. This artificially enhances the strength of the signal. It is not clear whether cross-linking of rituximab serves to speed up and strengthen the physiological effect of rituximab signaling so it can be measured in a short assay, or provides such a strong signal that non-physiologic changes are induced that may not be relevant clinically.

Sample processing itself induces signaling changes, making the study of signaling in primary lymphoma samples extremely difficult. To avoid this predicament, most signaling assays involve use of cell lines which are very different from primary malignant cells. Chronic lymphocytic leukemia (CLL) cells require considerably less manipulation than do lymphoma cells harvested from nodes, and signaling in CLL cells in response to rituximab has been reported (12).

In vitro synergy between rituximab and cytotoxic chemotherapy has been demonstrated (13). Clones grown to be resistant to rituximab in vitro also show resistance to cytotoxic chemotherapy suggesting common mechanisms may be at play (14). Vega and colleagues recently demonstrated that both intact rituximab and modified rituximab, that no longer fixes complement or mediates ADCC, inhibit the activity of cell survival/growth including those that regulate drug resistance. The modified antibodies sensitized lymphoma cells to both CDDP and Fas ligand-induced apoptosis (15). Evaluation of the effects of such combinations have been among the most valuable of in vitro assays exploring rituximab mechanisms of action, and are particularly relevant given clinical evidence that such combinations are effective.

Overall, in vitro studies suggest rituximab-induced signaling can contribute to the anti-tumor effect of therapy and can synergize with cytotoxic therapy.

1B. Animal model studies

Animal models carry the potential advantage of allowing for prolonged exposure of malignant cells to rituximab in an environment where the cells are growing in a physiologic environment. Synergy has been observed in animal models of rituximab and chemotherapy (16,17) and these studies have helped provide impetus for clinical evaluation of such combinations, however they add little to our understanding of rituximab mechanisms of action as either single agents or in combination with chemotherapy.

1C. Clinical studies

Indirect evidence suggests that anti-CD20 therapy can result in regression of B cell lymphoma in a manner that is not dependent on robust immune participation. The earliest such evidence comes from the first studies of radiolabeled anti-CD20 therapy. Subjects were preloaded with cold (unradiolabeled) antibody to improve biodistribution of the radio-labeled antibody. Unexpectedly, some subjects demonstrated shrinkage of lymphoma after the cold antibody but before the radiolabeled antibody was given(18). The cold antibody used in this study was a murine IgG2a (B1), demonstrating that even a murine IgG antibody, with its relatively poor ability to interact with human Fc receptors, can have anti-tumor activity.

Rituximab injected directly into the cerebrospinal fluid in patients with central nervous system lymphoma has been reported to have local anti-lymphoma effects(19). The lack of significant levels of complement or cells capable of mediating ADCC in the CNS would suggest a direct effect of rituximab on the malignant cells. On the other hand, a small study suggests the therapeutic effect of CNS administration of rituximab can be augmented by concomitant injection of serum into the CNS as a source of complement(20).

The overall concept that rituximab will enhance the efficacy of chemotherapy may seem counter-intuitive if one assumes that the mechanism of action of monoclonal antibody therapy is based on activation of immune effector mechanisms. However, clinical data in a variety of B cell malignancies provides strong evidence that rituximab and chemotherapy can work well together (3,4,21,22). One potential explanation for the success of these regimens that include immune effectors is that chemotherapy enhances the sensitivity of malignant B cells to immune mediated lysis. However, FcγRIIIA (CD16) and FcγRIIA (CD32) polymorphisms in follicular lymphoma patients treated with rituximab plus CHOP chemotherapy did not predict clinical outcome (23). This negative data provides circumstantial support for the hypothesis that direct signaling either induces apoptosis in chemo-sensitized cells or sensitizes the malignant cells to cytotoxic therapy when rituximab is given along with chemotherapy.

1D. Signaling induced cell death – Conclusions

Taken together, there is indirect but convincing evidence extending from in vitro analyses through clinical trial correlative studies that signaling effects of rituximab on B cells independent of additional immune effector mechanisms can contribute to the anti-tumor activity of rituximab. The strongest such evidence is the clear demonstration that the combination of rituximab and cytotoxic chemotherapy is effective in a variety of B cell malignancies. Indeed, many investigators are evaluating novel approaches to combining rituximab with cytotoxic chemotherapy in vitro, in animal models and in clinical trials based on the synergistic effects of rituximab signaling and cytotoxic therapy.

2. CMC

2A. In vitro studies

Several studies have demonstrated in vitro that rituximab is highly efficient at mediating CMC of various B cell lines as well as fresh malignant B cell samples. The expression of complement inhibitory molecules (CD55 and CD59) on malignant B cells correlates with the extent of in vitro lysis (24-29). Follicular lymphoma is more sensitive to rituximab clinically, and follicular lymphoma cells are more effectively lysed by complement in vitro when compared to cells from subjects with large cell lymphoma or mantle cell lymphoma (30). These studies generally utilize serum as a source of complement, which speaks to the importance of CMC in the circulation, but tells us little about whether complement plays a role in the anti-tumor activity of rituximab within lymph nodes or at other extravascular sites. To begin assessing this issue, we recently evaluated whether CMC is observed when rituximab is added to transudative

pleural fluid or ascites that served as surrogates for extravascular fluid. Although the concentration of various components of the complement cascade was lower in these fluids than in plasma, there is enough complement in these fluids to mediate CMC(31) and suggest CMC could be contributing to the anti-tumor activity of rituximab in the extravascular, as well as intravascular, compartments.

2B. Animal model studies

A number of in vivo tumor models suggest the anti-tumor activity of rituximab is dependent, at least in part, on complement (32,33). Depletion of cellular effectors in these models had no effect on the therapeutic response to rituximab, while depletion of complement through use of Cobra Venom Factor abolished the therapeutic response. However, these models involved use of rituximab (a chimeric humanized IgG) in mice which would require interaction between a murine target cell, human IgG and murine immune effector cell if ADCC were to take place. In addition, the target cells express low levels of complement inhibitor molecules. Complement activation was recently found to play a role in antibody-induced infusion toxicity in both a rodent model and non-human primates. Use of antibodies modified to have a reduced ability to fix complement induced fewer infusion reactions. In the rodent model, this reduction in infusion reaction had little effect on anti-tumor activity (34). These data support the contention that complement fixation contributes to infusion reactions, although they also confirm that infusion reactions are not good predictors of a therapeutic response to antibody.

2C. Clinical studies

Clinical observations demonstrate complement is activated during treatment with rituximab in some but not all patients(35) and that complement activation correlates with infusional toxicity (36). CLL cells that remain after rituximab treatment have been found to have a higher surface expression of the complement inhibitor CD59 when compared to pretherapy expression of this marker (37,38). This suggests selective elimination or trafficking out of the circulation of CLL cells expressing lower amounts of CD59 that might be more sensitive to CMC. However, no correlation has been found between expression of complement inhibitors and clinical response to rituximab treatment(29). Thus, complement consumption mediated by rituximab may not translate into anti-tumor efficacy.

2D. CMC – Conclusions

CMC can clearly result in rapid cell death of rituximab-coated target cells, and is a prime mechanism of action for antibody therapy in some animal models. There is considerable data suggesting CMC occurs in patients after rituximab therapy, and that complement fixation plays a role in infusion reactions. What is less clear is the degree to which CMC contributes to the clinical anti-tumor response to therapy, and whether it is active against cells that are outside the intravascular compartment. Overall, the evidence that CMC contributes to the clinical efficacy of rituximab has been seen as strong enough to encourage the clinical development of next generation anti-CD20 antibodies with an enhanced ability to fix complement (39), and infusion of plasma during rituximab treatment to replenish complement (40). Whether such approaches will be clinically superior to rituximab therapy remains to be seen.

3. ADCC

3A. In vitro studies

Monoclonal antibodies can induce ADCC mediated by a variety of effector cells including NK cells, granulocytes and macrophages (41,42). These processes require that the Fc of the antibody bound to the target cell bind to Fc γ receptor (Fc γ Rs) on the effector cells triggering immune cell activation and death of the target cell (43). Rituximab can induce ADCC of human

lymphoma cell lines by human peripheral blood mononuclear cells (24). However, in vitro detection of ADCC with rituximab and other antibodies often involves very high, non-physiologic, effector to target cell ratios – often in the 25-50:1 range. Immune effector cells are often pre-activated with cytokines such as IL2 prior to the ADCC assay. It is not clear whether these manipulations serve to speed up ADCC so it can be measured in a 4 hour time frame, or provide for killing via a mechanism that is not relevant clinically.

Most research exploring ADCC and rituximab has focused on interactions of rituximab with CD16 on NK cells; however, other Fc receptors including CD32 (25), CD64 (44) and CD89 (45), may also contribute to anti-CD20 mediated ADCC. Expression of these receptors, and ADCC mediated by cells expressing them, is enhanced by the presence of a number of different cytokines including IFN γ that can be produced by activated NK cells. Thus, it is conceivable that CD16 plays a role in activating NK cells locally, and that the resulting cytokines produced by NK cells enhances ADCC mediated by other receptors and other cells.

3B. Animal model studies

As outlined above, CMC is the central mechanism of action in some animal models of rituximab therapy. In others, CMC appears to play a limited role and ADCC is most important (46). Perhaps the strongest evidence for the importance of ADCC in animal models comes from the work of Clynes and colleagues who found that antibody was effective in wild type mice, but not in mice lacking the common Fc γ chain (47). Furthermore, the anti-tumor effect of mAb therapy was enhanced in mice lacking Fc γ RIIb, which is an inhibitory receptor (48), providing additional evidence that the interactions between the antibody and FcR is central to determining the efficacy of therapy.

3C. Clinical studies

The most convincing evidence that ADCC is mechanistically involved in clinical response to rituximab therapy comes from correlative studies demonstrating an association between polymorphisms on CD16 (also known as Fc γ RIIIa) and clinical response to single agent rituximab. CD16 homozygous for valine at 158 (VV) has a higher affinity for IgG1 than does CD16 with phenylalanine at that position (VF or FF) (49). Patients with follicular lymphoma with the VV genotype have a better clinical response to rituximab than patients that are VF or FF (50,51). The same polymorphism is also predictive of rituximab response in patients with Waldenström's macroglobulinemia (52). Follicular lymphoma patients homozygous for histidine at 131 (HH) on Fc γ RIIa, also have an improved response to rituximab (51). This data highlights the importance of Fc-Fc γ R interactions in the anti-tumor effects of rituximab and suggests that ADCC is a major mechanism of action. It also points towards NK cells, the principal cells that express CD16, as being key contributors to the anti-tumor activity of mAb. However, not all data indicate these polymorphisms are clinically relevant. CD16 polymorphisms did not predict clinical response to rituximab in chronic lymphocytic leukemia (CLL) patients (53) or follicular lymphoma patients treated with rituximab plus CHOP (23).

This strong, if indirect, evidence that the interaction between rituximab Fc and CD16 is central to the mechanisms of action of rituximab has led to the development and clinical evaluation of novel strategies for enhancing target cell lysis by NK cells including the addition to rituximab of immunostimulatory agents designed to activate NK cells (54,55), and development of anti-CD20 antibodies with stronger affinity for CD16 (43,56). Thus far, none of these strategies has been unequivocally successful, but evaluation is ongoing.

3D. Antibody dependent cellular cytotoxicity – Conclusions

In vitro, animal model and correlative clinical studies suggest that interaction of antibody Fc with CD16 contributes to the clinical anti-tumor activity of single agent rituximab. The most

obvious interpretation of these findings is that NK cells are mediating ADCC of rituximab-coated target cells. However, there are alternative explanations. For example, NK cells are activated and produce IFN γ when they come in contact with rituximab-coated target cells (57). This IFN γ could have direct anti-tumor effects on the malignant cells, or could be activating other immune effector cells that could contribute to ADCC. Indeed, IFN γ is known to upregulate the high affinity Fc γ receptor (CD64) on granulocytes (58), and so IFN γ could enhance their ability to mediate ADCC. Whether the contribution of NK cells is direct or indirect will be difficult to discern. However, clinical trials comparing rituximab to next generation anti-CD20 antibodies that have an enhanced ability to bind to Fc γ R and activate NK cells will help us understand the clinical importance of ADCC in the anti-tumor activity of rituximab.

4. Interacting mechanisms

The discussion above addresses the major mechanisms of action of rituximab independently. In fact, results of a number of studies point to more than one mechanism playing a role in a single model including an increase in complement inhibitory molecules, decreased expression of CD20, and enhanced expression of anti-apoptotic molecules (59,60).

There are also extensive interactions – both synergistic and antagonistic – between these mechanisms of action. A mechanism that may contribute to the anti-tumor activity of rituximab in one clinical scenario may actually inhibit the therapeutic response in another. Different mechanisms may contribute in different ways at different sites and times within an individual patient.

An excellent example is complement. The influence of the complement system on the immune response goes beyond traditional CMC. Complement can impact on B cell activation directly (61) and C3 has been shown to have a direct inhibitory effect on NK cell function (62). Clearance of apoptotic bodies by complement may impede development of an active immune response (63,64). This may help explain our apparently paradoxical finding of a correlation between polymorphisms in the C1q component of complement (A vs G at position 276) and duration of response to single agent rituximab (65). In this study, subjects with a polymorphism associated with decreased protein expression had a better outcome. More specifically, we found prolonged remission among subjects that were carriers of the A allele, which is associated with lower C1q levels and less biologic C1q activity, when compared to homozygous G subjects who have more biologically active C1q.

The presence of immune complexes on the surface of cells does not always lead to CMC. Taylor and colleagues have demonstrated that monocytes and macrophages may “shave” rituximab-complement complexes off the surface of CLL cells (66,67) resulting in viable malignant cells that no longer have surface antigen on their surface. Whether this phenomenon is limited to situations such as CLL where opsonized cells circulate through the liver and spleen, or also takes place in the lymph nodes, remains to be seen.

We recently reported that NK-cell activation and ADCC induced by rituximab-coated target cells are inhibited by the C3b component of complement (68). The epitope to which C3b binds to IgG Fc is very close to the epitope on the IgG Fc that binds to CD16. Thus, complement fixation may actually impede rituximab binding to CD16 and ADCC. In preliminary studies we have found that depletion of complement can actually enhance the efficacy of antibody therapy in a murine model (31).

Complement depletion, systemically or locally, could result in loss of CMC, or alternatively loss of the inhibitory effect of complement on other mechanisms (66). Taken together, CMC can result in anti-tumor activity in some situations, but this may be offset by shaving of the

immune complex from the surface of viable malignant cells, or inhibition of ADCC through C3b blockade of the interaction between rituximab and NK cells. Thus, complement may be a positive contributor to therapy in some circumstances, but have negative effects in others.

The dynamics of interaction between such mechanisms are extremely complex given the differences in microenvironment, saturation of receptors and possible exhaustion of proteins or cells vital for effective target cell destruction. This could well explain many of the apparently conflicting results discussed above. Rituximab concentration, immune effector cell infiltration, FcR saturation, and complement depletion in the local micro-environment could all impact on whether a given mechanism is active. Mechanisms that are important in one setting may be less important in another, even in the same patient at different times or locations.

Conclusion

The data presented above suggest that rituximab-mediated signaling, CMC, and ADCC all contribute to rituximab's anti-tumor activity. Given this complexity, where do we go from here? We are unable to say "This is it!" with respect to a single mechanism being central to clinical response to rituximab. However, the ongoing evaluation of mechanisms of action has led to new rituximab-based combinations, novel schedules, and the design of the next generation of antibodies discussed at length elsewhere in this volume. Preclinical validation of novel strategies is being followed by clinical evaluation of the most promising approaches. Clinical trials based on these data include evaluation of antibodies with an enhanced ability to signal, mediate CMC and mediate ADCC. Such studies need to be accompanied by rigorous correlative analysis, and will continue to be central to our ability to understand the importance of various mechanisms of action of rituximab in different settings. This knowledge can be used to improve on what is already an indispensable approach to therapy. The results related to an enhanced understanding of mechanisms of action could well be applicable beyond rituximab therapy of B cell malignancies and impact on treatment of other cancers and benign disorders.

Literature cited

1. Maloney DG, Grillo-Lopez AJ, Bodkin DJ, et al. IDEC-C2B8: results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma [see comments]. *J Clin Oncol* 1997;15(10):3266–74. [PubMed: 9336364]
2. McLaughlin P, Grillo-Lopez AJ, Link BK, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998;16(8):2825–33. [PubMed: 9704735]
3. Habermann TM, Weller EA, Morrison VA, et al. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. *J Clin Oncol* 2006;24(19):3121–7. [PubMed: 16754935]
4. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med* 2002;346(4):235–42. [PubMed: 11807147]
5. Maloney DG, Grillo-Lopez AJ, White CA, et al. Idec-C2b8 (Rituximab) Anti-Cd20 Monoclonal Antibody Therapy Patients With Relapsed Low-Grade Non-Hodgkins Lymphoma. *Blood* 1997;90(6):2188–2195. [PubMed: 9310469]
6. Egeblad M, Ewald AJ, Askautrud HA, et al. Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Dis Model Mech* 2008;1(23):155–67. discussion 165. [PubMed: 19048079]
7. Shan D, Ledbetter JA, Press OW. Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. *Cancer Immunol Immunother* 2000;48(12):673–83. [PubMed: 10752475]
8. Semac I, Palomba C, Kulangara K, et al. Anti-CD20 therapeutic antibody rituximab modifies the functional organization of rafts/microdomains of B lymphoma cells. *Cancer Res* 2003;63(2):534–40. [PubMed: 12543813]

9. Jazirehi AR, Gan XH, De Vos S, Emmanouilides C, Bonavida B. Rituximab (anti-CD20) selectively modifies Bcl-xL and apoptosis protease activating factor-1 (Apaf-1) expression and sensitizes human non-Hodgkin's lymphoma B cell lines to paclitaxel-induced apoptosis. *Mol Cancer Ther* 2003;2(11): 1183–93. [PubMed: 14617792]
10. Bonavida B. Rituximab-induced inhibition of antiapoptotic cell survival pathways: implications in chemo/immunosensitivity, rituximab unresponsiveness, prognostic and novel therapeutic interventions. *Oncogene* 2007;26(25):3629–36. [PubMed: 17530016]
11. Czuczman MS, Olejniczak S, Gowda A, et al. Acquisition of rituximab resistance in lymphoma cell lines is associated with both global CD20 gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels. *Clin Cancer Res* 2008;14(5):1561–70. [PubMed: 18316581]
12. Pedersen IM, Buhl AM, Klausen P, Geisler CH, Jurlander J. The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein-kinase-dependent mechanism. *Blood* 2002;99(4):1314–9. [PubMed: 11830481]
13. Emmanouilides C, Jazirehi AR, Bonavida B. Rituximab-mediated sensitization of B-non-Hodgkin's lymphoma (NHL) to cytotoxicity induced by paclitaxel, gemcitabine, and vinorelbine. *Cancer Biother Radiopharm* 2002;17(6):621–30. [PubMed: 12537665]
14. Jazirehi AR, Vega MI, Bonavida B. Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy. *Cancer Res* 2007;67(3):1270–81. [PubMed: 17283164]
15. Vega MI, Huerta-Yepez S, Martinez-Paniagua M, et al. Rituximab-mediated cell signaling and chemo/immuno-sensitization of drug-resistant B-NHL is independent of its Fc functions. *Clin Cancer Res* 2009;15(21):6582–94. [PubMed: 19861448]
16. Schliemann C, Palumbo A, Zuberbuhler K, et al. Complete eradication of human B-cell lymphoma xenografts using rituximab in combination with the immunocytokine L19-IL2. *Blood* 2009;113(10): 2275–83. [PubMed: 19005180]
17. Mohammad RM, Aboukameel A, Nabha S, Ibrahim D, Al-Katib A. Rituximab, Cyclophosphamide, Dexamethasone (RCD) regimen induces cure in WSU-WM xenograft model and a partial remission in previously treated Waldenstrom's macroglobulinemia patient. *J Drug Target* 2002;10(5):405–10. [PubMed: 12442811]
18. Kaminski MS, Zasadny KR, Francis IR, et al. Radioimmunotherapy of B-cell lymphoma with [131I] anti-B1 (anti-CD20) antibody. *N Engl J Med* 1993;329(7):459–65. [PubMed: 7687326]
19. Rubenstein JL, Fridlyand J, Abrey L, et al. Phase I study of intraventricular administration of rituximab in patients with recurrent CNS and intraocular lymphoma. *J Clin Oncol* 2007;25(11):1350–6. [PubMed: 17312328]
20. Takami A, Hayashi T, Kita D, Nishimura R, Asakura H, Nakao S. Treatment of primary central nervous system lymphoma with induction of complement-dependent cytotoxicity by intraventricular administration of autologous-serum-supplemented rituximab. *Cancer Sci* 2006;97(1):80–3. [PubMed: 16367925]
21. Czuczman MS, Grillo-Lopez AJ, White CA, et al. Treatment of patients with low-grade B-cell lymphoma with the combination of chimeric anti-CD20 monoclonal antibody and CHOP chemotherapy. *J Clin Oncol* 1999;17(1):268–76. [PubMed: 10458242]
22. Keating MJ, O'Brien S, Albitar M, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. *J Clin Oncol* 2005;23(18):4079–88. [PubMed: 15767648]
23. Carlotti E, Palumbo GA, Oldani E, et al. FcγRIIIA and FcγRIIA polymorphisms do not predict clinical outcome of follicular non-Hodgkin's lymphoma patients treated with sequential CHOP and rituximab. *Haematologica* 2007;92(8):1127–30. [PubMed: 17650444]
24. Reff ME, Carner K, Chambers KS, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 1994;83(2):435–45. [PubMed: 7506951]
25. Flieger D, Renoth S, Beier I, Sauerbruch T, Schmidt-Wolf I. Mechanism of cytotoxicity induced by chimeric mouse human monoclonal antibody IDEC-C2B8 in CD20-expressing lymphoma cell lines. *Cell Immunol* 2000;204(1):55–63. [PubMed: 11006018]

26. Golay J, Lazzari M, Facchinetti V, et al. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 2001;98(12):3383–9. [PubMed: 11719378]
27. Harjunpaa A, Junnikkala S, Meri S. Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. *Scand J Immunol* 2000;51(6):634–41. [PubMed: 10849376]
28. Bellosillo B, Villamor N, Lopez-Guillermo A, et al. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood* 2001;98(9):2771–7. [PubMed: 11675350]
29. Weng WK, Levy R. Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. *Blood* 2001;98(5):1352–7. [PubMed: 11520782]
30. Manches O, Lui G, Chaperot L, et al. In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 2003;101(3):949–54. [PubMed: 12393572]
31. Wang S-Y, Cagley J, Fritzinger D, Vogel C-W, John WS, Weiner GJ. Depletion of the C3 Component of Complement Enhances the Ability of Rituximab-Coated Target Cells to Activate Human NK Cells and Improves the Efficacy of Monoclonal Antibody Therapy in An in Vivo Model 112:2008–3620.
32. Di Gaetano N, Cittera E, Nota R, et al. Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol* 2003;171(3):1581–7. [PubMed: 12874252]
33. Golay J, Cittera E, Di Gaetano N, et al. The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. *Haematologica* 2006;91(2):176–83. [PubMed: 16461301]
34. Tawara T, Hasegawa K, Sugiura Y, et al. Complement activation plays a key role in antibody-induced infusion toxicity in monkeys and rats. *J Immunol* 2008;180(4):2294–8. [PubMed: 18250438]
35. Zhou X, Hu W, Qin X. The role of complement in the mechanism of action of rituximab for B-cell lymphoma: implications for therapy. *Oncologist* 2008;13(9):954–66. [PubMed: 18779537]
36. van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. Complement activation plays a key role in the side-effects of rituximab treatment. *Br J Haematol* 2001;115(4):807–11. [PubMed: 11843813]
37. Bannerji R, Kitada S, Flinn IW, et al. Apoptotic-regulatory and complement-protecting protein expression in chronic lymphocytic leukemia: relationship to in vivo rituximab resistance. *J Clin Oncol* 2003;21(8):1466–71. [PubMed: 12697868]
38. Treon SP, Mitsiades C, Mitsiades N, et al. Tumor Cell Expression of CD59 Is Associated With Resistance to CD20 Serotherapy in Patients With B-Cell Malignancies. *J Immunother* 2001;24(3):263–271.
39. Pawluczak AW, Beurskens FJ, Beum PV, et al. Binding of submaximal C1q promotes complement-dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or rituximab (RTX): considerably higher levels of CDC are induced by OFA than by RTX. *J Immunol* 2009;183(1):749–58. [PubMed: 19535640]
40. Klepfish A, Schattner A, Ghoti H, Rachmilewitz EA. Addition of fresh frozen plasma as a source of complement to rituximab in advanced chronic lymphocytic leukaemia. *Lancet Oncol* 2007;8(4):361–2. [PubMed: 17395110]
41. Dall'Ozzo S, Tartas S, Piantaud G, et al. Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. *Cancer Res* 2004;64(13):4664–9. [PubMed: 15231679]
42. Lefebvre ML, Krause SW, Salcedo M, Nardin A. Ex vivo-activated human macrophages kill chronic lymphocytic leukemia cells in the presence of rituximab: mechanism of antibody-dependent cellular cytotoxicity and impact of human serum. *J Immunother* 2006;29(4):388–97. [PubMed: 16799334]
43. Bowles JA, Wang SY, Link BK, et al. Anti-CD20 monoclonal antibody with enhanced affinity for CD16 activates NK cells at lower concentrations and more effectively than rituximab. *Blood*. 2006
44. Hernandez-Ilizaliturri FJ, Jupudy V, Ostberg J, et al. Neutrophils contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. *Clin Cancer Res* 2003;9(16 Pt 1):5866–73. [PubMed: 14676108]

45. Stockmeyer B, Dechant M, van Egmond M, et al. Triggering Fc alpha-receptor I (CD89) recruits neutrophils as effector cells for CD20-directed antibody therapy. *J Immunol* 2000;165(10):5954–61. [PubMed: 11067958]
46. Wooldridge JE, Ballas Z, Krieg AM, Weiner GJ. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood* 1997;89(8):2994–8. [PubMed: 9108420]
47. Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV. Fc receptors are required in passive and active immunity to melanoma. *Proc Natl Acad Sci U S A* 1998;95(2):652–6. [PubMed: 9435247]
48. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med* 2000;6(4):443–6. [PubMed: 10742152]
49. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood* 1997;90(3):1109–14. [PubMed: 9242542]
50. Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* 2002;99(3):754–8. [PubMed: 11806974]
51. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 2003;21(21):3940–7. [PubMed: 12975461]
52. Treon SP, Hansen M, Branagan AR, et al. Polymorphisms in FcgammaRIIIA (CD16) receptor expression are associated with clinical response to rituximab in Waldenstrom's macroglobulinemia. *J Clin Oncol* 2005;23(3):474–81. [PubMed: 15659493]
53. Farag SS, Flinn IW, Modali R, Lehman TA, Young D, Byrd JC. Fc gamma RIIa and Fc gamma RIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. *Blood* 2004;103(4):1472–4. [PubMed: 14563637]
54. Friedberg JW, Neuberg D, Gribben JG, et al. Combination immunotherapy with rituximab and interleukin 2 in patients with relapsed or refractory follicular non-Hodgkin's lymphoma. *Br J Haematol* 2002;117(4):828–34. [PubMed: 12060117]
55. Leonard JP, Link BK, Emmanouilides C, et al. Phase I Trial of Toll-Like Receptor 9 Agonist PF-3512676 with and Following Rituximab in Patients with Recurrent Indolent and Aggressive Non Hodgkin's Lymphoma. *Clin Cancer Res* 2007;13(20):6168–74. [PubMed: 17947483]
56. Robak T. GA-101, a third-generation, humanized and glyco-engineered anti-CD20 mAb for the treatment of B-cell lymphoid malignancies. *Curr Opin Investig Drugs* 2009;10(6):588–96.
57. Bowles JA, Weiner GJ. CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. *Journal of Immunological Methods* 2005;304:88–99. [PubMed: 16109421]
58. Hoffmeyer F, Witte K, Schmidt RE. The high-affinity Fc gamma RI on PMN: regulation of expression and signal transduction. *Immunology* 1997;92(4):544–52. [PubMed: 9497497]
59. Dalle S, Dupire S, Brunet-Manquat S, Reslan L, Plesa A, Dumontet C. In vivo model of follicular lymphoma resistant to rituximab. *Clin Cancer Res* 2009;15(3):851–7. [PubMed: 19188155]
60. Stolz C, Hess G, Hahnel PS, et al. Targeting Bcl-2 family proteins modulates the sensitivity of B-cell lymphoma to rituximab-induced apoptosis. *Blood* 2008;112(8):3312–21. [PubMed: 18689543]
61. Carroll MC. The complement system in B cell regulation. *Mol Immunol* 2004;41(23):141–6. [PubMed: 15159059]
62. Charriaut C, Senik A, Kolb JP, Barel M, Frade R. Inhibition of in vitro natural killer activity by the third component of complement: role for the C3a fragment. *Proc Natl Acad Sci U S A* 1982;79(19):6003–7. [PubMed: 6985269]
63. Boackle SA. Complement and autoimmunity. *Biomed Pharmacother* 2003;57(7):269–73. [PubMed: 14499171]
64. Botto M, Walport MJ. C1q, autoimmunity and apoptosis. *Immunobiology* 2002;205(45):395–406. [PubMed: 12396002]
65. Racila E, Link BK, Weng WK, et al. A Polymorphism in the Complement Component C1qA Correlates with Prolonged Response Following Rituximab Therapy of Follicular Lymphoma. *Clin Cancer Res* 2008;14(20):6697–703. [PubMed: 18927313]

66. Kennedy AD, Beum PV, Solga MD, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol* 2004;172(5):3280–8. [PubMed: 14978136]
67. Beum PV, Lindorfer MA, Taylor RP. Within peripheral blood mononuclear cells, antibody-dependent cellular cytotoxicity of rituximab-opsonized Daudi cells is promoted by NK cells and inhibited by monocytes due to shaving. *J Immunol* 2008;181(4):2916–24. [PubMed: 18684983]
68. Wang SY, Racila E, Taylor RP, Weiner GJ. NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. *Blood* 2008;111(3):1456–63. [PubMed: 18024795]

Figure 1.

A) Mechanisms of action related to direct rituximab-induced signaling

B) Mechanisms of action related to rituximab-induced CMC and ADCC with potential that these two mechanisms can be antagonistic.