[Frontiers in Bioscience 16, 277-306, January 1, 2011]

Molecular mechanisms of the antitumor effects of anti-CD20 antibodies

Magdalena Winiarska[1], Eliza Glodkowska-Mrowka[1], Jacek Bil[1], Jakub Golab[1,2]

[1]Department of Immunology, Centre of Biostructure Research, Medical University of Warsaw, Banacha 1A F building, 02-097 Warsaw, Poland, [2]Institute of Physical Chemistry, Polish Academy of Sciences, Department 3, 01-224 Warsaw, Poland

TABLE OF CONTENTS

1. ABSTRACT

Anti-CD20 monoclonal antibodies (mAbs) have become the mainstay in the treatment of non-Hodgkin's lymphomas and have shown significant activity in patients with B-cell chronic lymphocytic leukemia. Antitumor action of these antibodies results from triggering of indirect effector mechanisms of the immune system that include activation of complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), or phagocytosis. Moreover, some studies indicate direct influence of anti-CD20 mAbs on tumor cells that leads to induction of various types of cell death. Despite the wealth of data on the mechanisms of cytotoxicity that accumulated over the last two decades their relative contribution to the therapeutic outcome is still difficult to predict in individual patients. Elucidation of molecular mechanisms of anti-CD20 mAbs action is necessary to deliver their maximal activity in rationally deigned combinations with other therapeutic approaches and to design next generation anti-CD20 mAb with improved ability to eliminate tumor cells.

2. INTRODUCTION

The idea of using antibodies as antitumor therapeutics is not new. The original Paul Ehrlich's concept of "magic bullet" that would eliminate diseased cells while sparing normal tissues inspired many generations of researchers looking for disease-selective therapeutics. "Magic bullets" became feasible with the development of hybridoma technology that enabled production of monoclonal antibodies (mAbs) in quantities sufficient for

Table 1. Monoclonal antibodies approved for clinical use in oncology

mAb name	Target antigen	Used to treat	Clinical approval
Rituximab	CD20	Non-hodgkin lymphoma	1997
Ibritumomab tiuxetan	CD20	Non-hodgkin lymphoma	2002
Tositumomab	CD20	Non-hodgkin lymphoma	2003
Ofatumumab	CD20	Chronic lymphocytic leukemia	2009
Alemtuzumab	CD52	Chronic lymphocytic leukemia	2001
Gemtuzumab ozogamicin	CD33	Acute myelogenous leukemia	2000
Bevacizumab	VEGF	Colorectal cancer Non-small cell lung cancer Breast cancer Glioblastoma Kidney cancer	2004 2006 2008 2009 2009
Panitumumab	EGFR	Colorectal cancer	2006
Cetuximab	EGFR	Colorectal cancer Head & neck cancers	2004 2006
Trastuzumab	HER2	Breast cancer	1998

Table 2. Anti-CD20 monoclonal antibodies

Antibody	Clinical status	Characteristics			effector mechanisms (compared with rituximab)		
		Origin	Type	Isotype	CDC	ADCC	Apoptosis
Rituximab	Approved	Chimeric	I	IgG1	=	=	=
Tositumomab*	Approved	Murine	II	IgG2a	-	=	+++
Ofatumumab	Approved	Human	I	IgG1	+++	=	=
Ibritumomab**	Approved	Murine	***	IgG1	***	***	***
Ocrelizumab	Phase 3	Humanized	I	IgG1	=	+	=
Veltuzumab	Phase 2	Humanized	I	IgG1	+	=	=
Obinutuzumab	Phase 2	Humanized	II	IgG1	-	+++	+++
PRO131921	Phase 2	Humanized	I	IgG1	+	++	=
AME-133	Phase 2	Humanized	I	IgG1	=	+	=
LFB-R603/EMAB-6	Phase 1	Chimeric	I	IgG1	=	+++	=

* radioimmunoconjugate bound to ^{131}iodine, ** radioimmunoconjugate bound to ^{90}ytrium, *** no data available

clinical use (1). MAbs themselves proved to be the tools that enabled identification of numerous tumor-associated antigens that served as targets for "magic bullet". Moreover, mAbs are used in diagnostic procedures that are currently employed to delineate the antigenic profile of tumor cells thereby determining treatment to be utilized in a particular situation.

Currently 10 mAbs have been approved for clinical use in oncology (Table 1). Four of these target a single antigen - a CD20 molecule expressed by normal B cells and several types of tumor cells. CD20 became the first identified molecule targeted by a clinically approved mAb - rituximab. At least 6 additional anti-CD20 antibodies are either approved for clinical use or are in various stages of clinical development (Table 2).

During the 13 years since its clinical approval in 1997 rituximab has significantly improved response rates and patients survival in a variety of lymphoid malignancies. In developed countries almost all patients diagnosed with CD20-positive B cell non-Hodgkin's lymphoma (NHL) are treated with anti-CD20 mAbs. Initial clinical studies indicated that rituximab alone can induce objective, albeit mainly partial antitumor responses in approximately 50% of patients. Complete responses and long-term survival is observed more frequently in patients treated with rituximab and conventional chemotherapeutics in several combination schedules, such as R-CHOP (rituximab + cyclophosphamide, doxorubicin, vincristine and prednisone) or R-CVP (rituximab + cyclophosphamide, vincristine and prednisone). In addition, rituximab proved to be effective and safe in the management of a number of autoimmune diseases and demonstrated utility in transplantation (2-3). Because of all these favorable effects in multiple different diseases rituximab has been coined a nickname of "vitamin R" (4). However, despite continued progress in dissecting the molecular mechanisms of action of rituximab and other anti-CD20 mAbs we still face a number of unanswered questions. Elucidation of these mechanisms is vital for improving currently used therapeutic regimens.

3. WHY ANTI-CD20 MABS ARE SO EFFECTIVE?

It is widely accepted that rituximab as well as other anti-CD20 mAbs can trigger antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CDC), and direct induction of cell death but the relative contribution of these effector mechanisms has so far been difficult to pinpoint. Additionally, sensitization of tumor cells to conventional chemotherapeutics and induction of delayed vaccinal effects contribute to the clinical efficacy of these antibodies. CD20 molecules are very abundant in the plasma membrane of NHL cells, frequently exceeding 25 x 10^4 molecules per cell (which is 2-10-fold higher than CD19 (5)), allowing clustered opsonization of tumor cells by mAbs. Tight association of CD20 with plasma membrane results in binding of mAbs in a close proximity to the cell surface, which is important in deposition of C3/C5 convertases of the complement system and formation of the membrane attack complex (MAC) (6). Moreover, CD20 molecules tend to form complexes of at least 4 molecules (tetramers), which might facilitate formation of supramolecular clusters of mAbs that can stably bind C1q components of complement system (7-8).

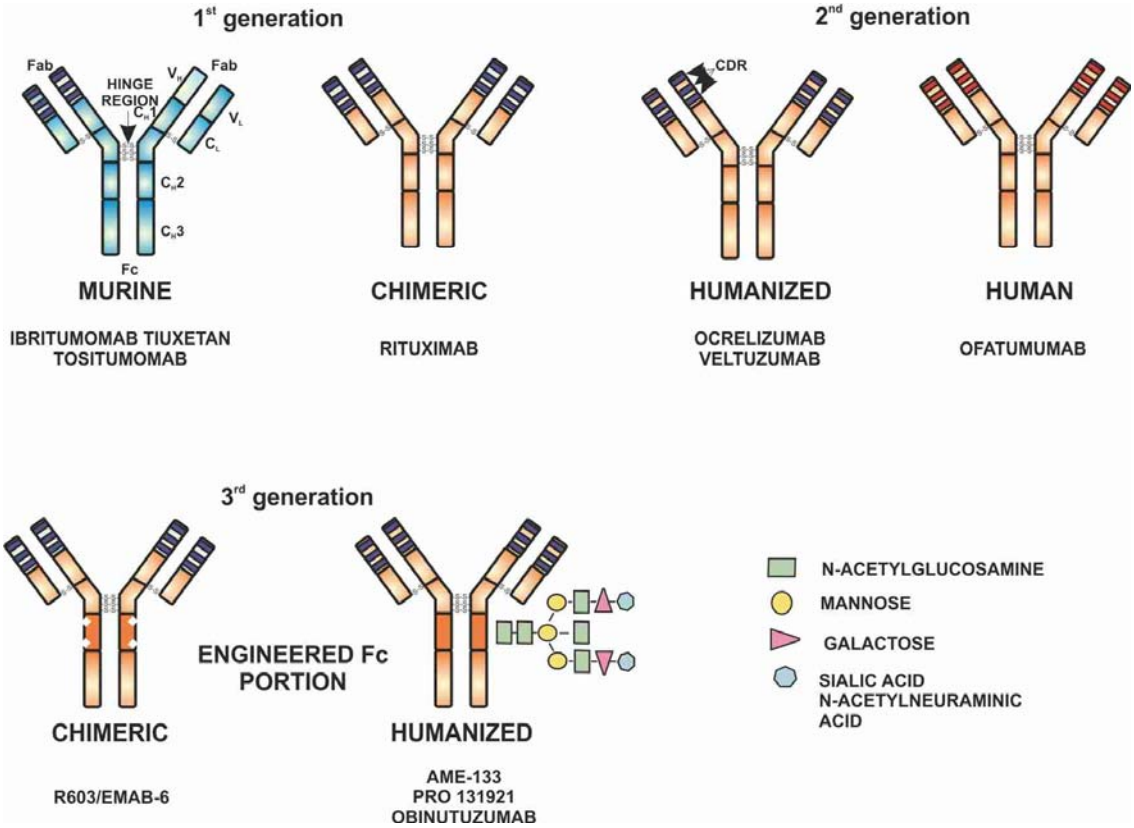

Figure 1. Three generations anti-CD20 monoclonal antibodies.

It appears that no CD20 ligand, which might interfere with anti-CD20 mAb binding, exists. CD20 is usually described as an antigen resistant to internalization or shedding even after mAb binding, thereby allowing for extended (up to several days) binding of mAb, a time which is more than enough for triggering effector mechanisms of FcγR-bearing cells of the immune system. Intriguingly, an increasing number of studies indicate that this is not always the case and several mechanisms of CD20 modulation exist that affect clinical efficacy of anti-CD20 mAbs (see below).

4. ANTIBODIES AND MOLECULES TARGETING CD20

The clinical success of rituximab, tositumomab and ibritumomab tiuxetan, which constitute a first generation of anti-CD20 mAbs, led to a design and evaluation of a number of other anti-CD20 therapeutics. These antibodies, classified as a second or a third generation anti-CD20 mAbs, can bind to distinct epitopes within CD20, have different pharmacokinetics and may trigger stronger effector mechanisms as compared with rituximab (Figure 1). Second generation antibodies have a humanized (ocrelizumab, veltuzumab) or a completely human (ofatumumab) IgG1 constant fragments, while third generation antibodies (PRO131921, AME-133, obinutuzumab, LFB-R603/EMAB-6) have humanized tails, additionally adjusted by genetic engineering to outperform rituximab in triggering effector mechanisms (9-12). Additionally, TRU-015, a small modular immunopharmaceutical composed of single chain anti-CD20 Fv fragment linked to human IgG1 CH2 and CH3 domains, which is undergoing clinical evaluation in the treatment of rheumatoid arthritis, has been shown to exert antitumor activity (13). The only second generation mAb approved for clinical use so far is ofatumumab, which is a more potent complement activator than rituximab (14). The likely reason for this is that ofatumumab binds to a distinct epitope than rituximab, which is located even closer to a B cell membrane. Moreover, ofatumumab seems to be more effective in triggering NK cell-mediated ADCC, which is independent of FcγRIIIa polymorphism (see below).

5. CD20 - A TARGET FOR RITUXIMAB

CD20 is an integral membrane molecule broadly expressed during B-cell ontogeny. It is present on the surface of numerous developmental stages of B cells from the early pre-B to the mature B cell stage (Figure 2). Its expression is ceased in normal as well as in malignant plasma cells, although one study reported that IFN-γ can induce CD20 expression in multiple myeloma cells (15). This restricted profile of CD20 expression ensures that neither B-cell precursors nor other cell lineages are endangered during anti-CD20 mAb treatment (16). Lack of significant influence on plasma cells should not lead to impaired immunoglobulin production against pathogens. CD20 is a non-glycosylated phosphoprotein expressed in

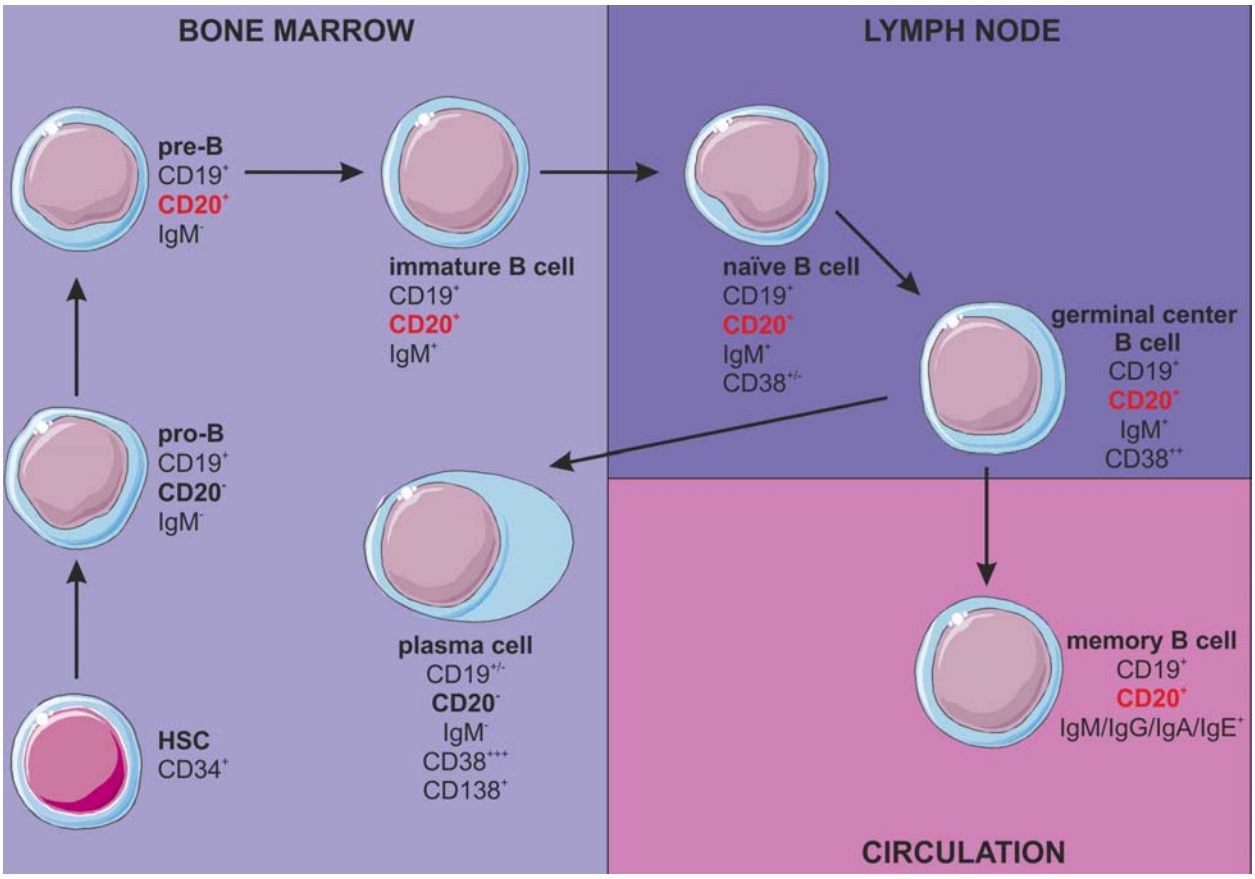

Figure 2. Structure of CD20 molecule.

three isoforms of 33, 35 or 37 kDa, depending on the degree of phosphorylation (17). It was initially described as a human B-lymphocyte specific antigen and was initially referred to as B-lymphocyte surface antigen B1 (18). Later studies indicated that not only B lymphocytes but also other types of cells including normal and malignant T cells (19-21) as well as melanoma stem cells (22) or blood-derived CD133-positive stem cells (23) are able to express CD20, although to a much lower levels than cells of the B-cell lineage.

5.1. Gene structure and expression

CD20 was the first identified and is the best studied member of the membrane-spanning 4A gene family (MS4A). The members of this family consisting of more than 25 proteins, such as high affinity IgE receptor FcεRIβ (MS4A2) and hematopoietic cell-specific protein (HTm4, MS4A3) are characterized by common structural features and similar gene structure with 20-40% amino acid homology (24). In 1988 Tedder *et al.* completely cloned the genes for human (25) and murine (26) CD20. Human 16-kb gene, consisting of 8 exons, is located on chromosome 11q12-q13, mapping to the same chromosomal location as the genes for 8 other members of MS4A family.

The CD20 gene does not contain a classical TATA box but has several minor transcription initiation sites. Consequently, the transcription may start at several points in exon 1 and 2 resulting in production of few different mRNAs, varying in length from 2.6 to 3.4 kb (25). Recently, a novel ΔCD20 mRNA transcript has been described that encodes a truncated protein of 15-17 kDa (27). This protein apparently associates with intracellular domains of normal CD20 and its levels increase in rituximab-resistant cells. It is possible that association with ΔCD20 can modulate the levels of CD20 in the plasma membrane or may affect translocation to lipid rafts.

CD20 expression is regulated by several transcription factors. The major region responsible for promoter activity is estimated to be located between -40 and -450 bp, containing at least four regulatory regions. The early studies led to the identification of several positive and negative regulatory *cis* elements in the sequence of the cloned gene (28). Subsequently, a BAT box containing an octamer binding site for commonly expressed Oct-1 and B-cell specific Oct-2 transcription factors at -255 to -201 was identified (29-30). As mutations of this site do not completely ablate CD20 expression (30), the involvement of other regions and transcription factors was suspected. Later on, it was shown that PU.1/Pip, important regulators of B-cell specific genes encoding such proteins as CD72 and immunoglobulin light chain, are able to bind to the CD20 promoter at -160 position and are involved in CD20 expression regulation (31). Additionally, a binding site for basic helix-loop-helix-zipper (bHLHZ) family of

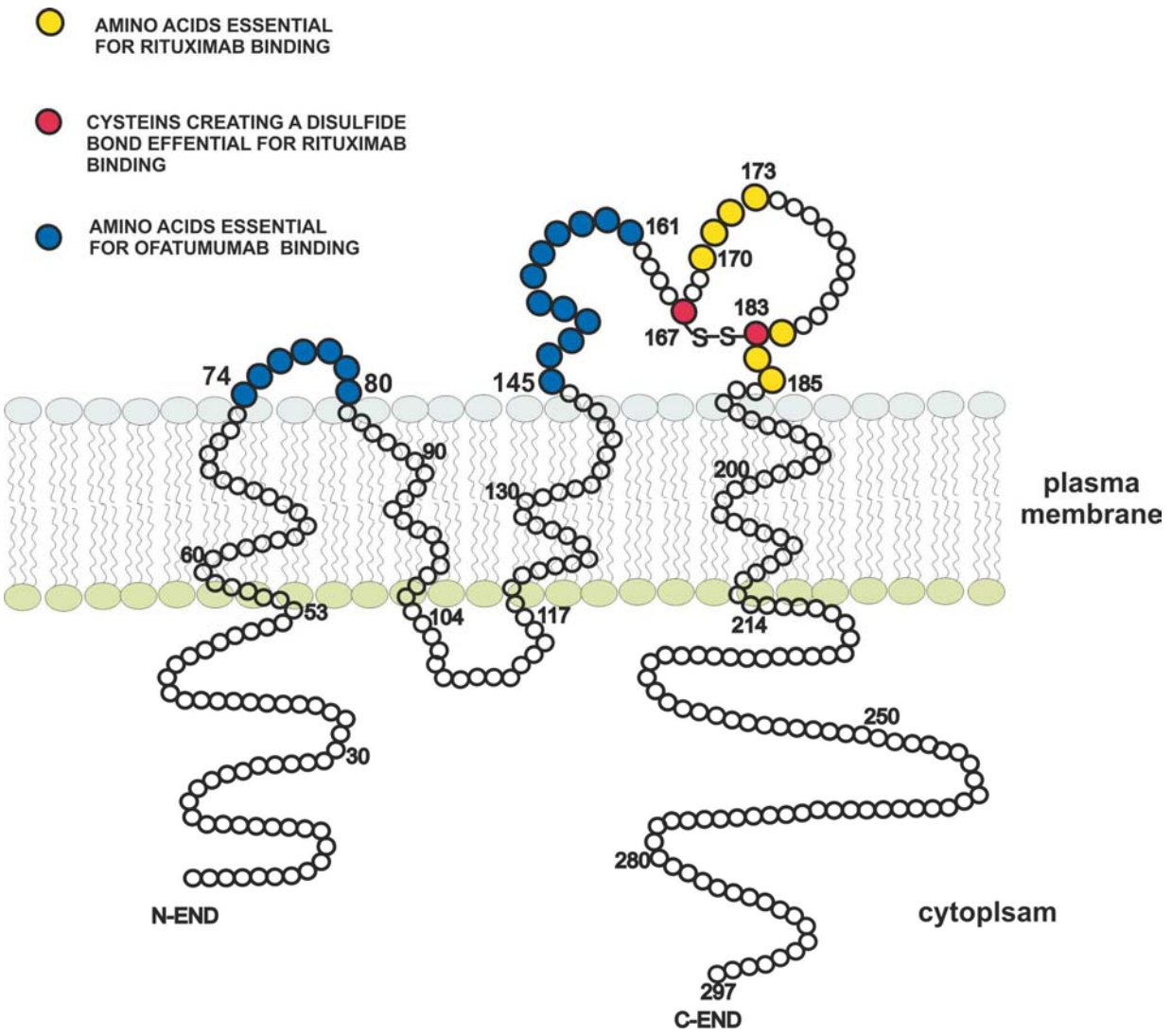

Figure 3. Expression of CD20 during B-cell development.

transcription factors, that bind ubiquitously expressed transcription factors and include TFE3 (transcription factor E3) and USF (upstream stimulatory factor) was found (31). Finally, another regulatory site in CD20 promoter containing a sequence for binding of the B cell-specific activator protein (BSAP), a regulatory protein encoded by Pax5 gene, was described (32).

The mechanisms underlying the regulation of CD20 expression still remain poorly understood. Several studies demonstrated that some cytokines, including interleukin-4 (IL-4) (33-34), granulocyte-macrophage colony stimulating unit (GM-CSF) (34), tumor necrosis factor (TNF) (34-35), interferon α (IFN-α) (36), and IFN-γ (15) are able to induce CD20 expression. Reactive oxygen species (ROS) may also influence CD20 expression (37). In CD20-positive cell lines irradiation or administration of hydrogen peroxide as a source of ROS has been reported to increase CD20 expression, while administration of antioxidants reversed this process (37).

5.2. Protein structure and localization

CD20 contains four membrane spanning domains with both termini in the cytoplasm (Figure 3) (38). Apart from the membrane-spanning domains, the protein contains two extracellular loops: a short (7 amino acids) loop localized between the first and the second transmembrane domains, that is highly conserved among the other MS4A family members, and a longer one (44 amino acids) localized between the third and the fourth transmembrane domains (39). Tight association with the plasma membrane protects the antigen from shedding.

As a hydrophobic, transmembrane protein, CD20 is constitutively associated with low affinity to lipid rafts (40-42). Binding of some (but not all) antibodies to CD20 increases its association with lipid rafts, favoring translocation of the molecule from the border to a centre of a raft area and facilitating oligomerization of CD20 molecules (42). At least in some cells CD20 appears to be localized to membrane protrusions known as microvilli

(43). Although CD20 co-localizes with BCR within lipid rafts on the surface of B-cells these molecules dissociate rapidly before BCR internalization (43). Additionally, CD20 has been shown to co-localize with CD40, MHC class I and II molecules, other tetraspan molecules (CD53, CD81 and CD82), and a transmembrane adapter protein p75/80 (also known as C-terminal Src kinase-binding protein, Cbp) (44-46). It is still not clear how CD20 interacts with these molecules.

5.3. CD20 function

Despite extensive ongoing research a natural ligand for CD20 has not been identified and the detailed function of the molecule remains to be elucidated. CD20 seems to be involved in B-cell development, activation and proliferation at least to some extent through regulation of intracellular calcium concentration and redistribution (7, 47). The majority of knowledge on the biological function of CD20 is based on studies with knockout animals, genetically modified cells or various monoclonal antibodies that ligate CD20 on normal or malignant B cells. The results of these studies, depending on the type of antibody used, have demonstrated that CD20 engagement may lead to increased B-cell survival (48) and proliferation (49) or, just the opposite, to cell cycle arrest (50) and induction of apoptosis (51-53).

The oligomeric structure and close homology to FcεRIβ, which is involved in Ca^{2+} conductance, suggest that CD20 may form a membrane channel involved in the regulation of ion transport. Indeed, initial studies revealed that transfection of CD20-negative cells with *cd20* gene increases cytoplasmic Ca^{2+} concentrations (7). However, these studies involved whole cell patch clamp analyses rather than cytosolic Ca^{2+} measurements, and more recent studies clearly indicated that CD20 is not a Ca^{2+} channel in transfected cells (54). Subsequently, Li *et al.* (55) have reported that human CD20 is responsible for calcium flux during BCR signaling. They suggested that CD20 is involved in extracellular calcium influx required during B-cell activation and works as store- or capacitance-operated calcium channel. These observations have been extended by Walshe *et al.*, who demonstrated that binding of rituximab to CD20 leads to its clustering with BCR followed by "borrowing" of the BCR signaling pathways that trigger Ca^{2+} release from intracellular stores (54). Even though the data presented above is convincing the relatively mild phenotype of CD20 knockout mice suggest that apart from CD20 also some other calcium channels may participate in B-cell activation.

Although the studies in CD20 knockout mice have surprisingly shown no significant impairment in humoral response (56), a recent report on the patient with CD20 deficiency has changed the general view on the function of CD20. The authors demonstrated that despite normal development of naïve B-cells, the absence of CD20 expression leads to immunodeficiency with severely impaired antibody formation, development of hypogammaglobulinemia, and impaired responses against polysaccharides after vaccination (57).

5.4. The epitopes recognized by anti-CD20 mAbs

The specific epitope for rituximab is encoded by sequences found in exon 5 of the MS4A1 gene, corresponding to a large extracellular domain comprising amino acids 165 to 184. Two amino acid sequences: ANPS and YCYSI at positions 170 to 173 and 182 to 185 are critical for rituximab binding (8, 58-59) (Figure 3). Therefore, the epitope is discontinuous and a disulfide bond between C167 and C183 is necessary to hold the two fragments in steric proximity (39). Different but overlapping epitopes are bound by ocrelizumab and other humanized antibodies. On the other hand ofatumumab binds to a completely different but also discontinuous epitope formed by sequences in a shorter (residues 74-80) and a longer loop (residues 145-161) of CD20 (60). The epitope for tositumomab has not been characterized in detail, but seems to be different than those recognized by rituximab and ofatumumab (61).

6. EFFECTOR MECHANISMS OF ANTI-CD20 MABS

Anti-CD20 mAbs can be divided into two types (62-63). Type I antibodies (rituximab and ofatumumab) redistribute CD20 to lipid rafts and are efficient activators of the complement cascade, but are relatively poor inducers of apoptosis, unless cross-linked by secondary anti-IgG antibodies (see below). Type II antibodies (tositumomab) are ineffective in complement activation, but readily activate homotypic adhesion of tumor cells and trigger a apoptotic or non-apoptotic cell death. Both types of antibodies can engage immune cells in ADCC or immunophagocytosis (62-64). The reasons for the differential engagement of effector mechanisms most likely lies in the unique specificities of type I vs. type II antibodies. This was most clearly demonstrated in studies that compared activation of CDC, phagocytosis or the capacity to induce cell death by engineered type I and II antibodies that had exactly the same Fc fragments of murine IgG2a subclass (61). The engineered mAbs retained the properties of original antibodies.

It is possible that it is the ability of type I antibodies to redistribute CD20 molecules to lipid rafts that makes these antibodies so effective in complement activation (62-63, 65). Lipid rafts are small (10-200 nm) membrane domains rich in cholesterol and glycosphingolipids, which facilitate the assembly of signaling molecules (66). It is conceivable that by concentrating multiple copies of CD20-mAb complexes within a clustered area of plasma membrane facilitates juxtaposition of Fc fragments allowing facile binding of C1q globular heads. Also the high cholesterol content within lipid rafts might contribute to efficient incorporation of MAC in the plasma membranes as has been demonstrated for erythrocytes (67).

For several reasons the relative contribution of each of these mechanism is difficult to predict. Most of the mechanistic studies have been done in *in vitro* systems, which do not include multiple variables affecting their efficacy in a living organism, including hyperoxia, which is

inherent to *in vitro* studies, lack of donor-derived serum, changes in the temperature during cell manipulation etc. Effector cells used in these experiments are not syngeneic, and the polymorphic differences between different donors are usually not considered. Moreover, the effector cells are obtained from normal, and not tumor-bearing individuals. Finally, the experimental assays usually take hours to days, but not weeks as is the case of anti-CD20 therapy in patients. Therefore, it is difficult if not even impossible to directly compare effector mechanisms in cell culture assays. Another important question is whether these assays should not consider simultaneous activation of all potential effector mechanisms that never act separately in patients.

Animal studies with anti-CD20 mAbs provide more valuable information, but there are additional variables inherent to species-specificities that affect interpretation of experimental data. For example, rituximab cannot be used in studies with normal mice since it does not bind to murine CD20 (68). This obstacle can be obviated by using either transgenic mice with human CD20, syngeneic models with murine tumor cells transfected with human CD20, or immunodeficient mice transplanted with human tumor cells. However, it is hard to predict an extent of species-dependent specificity in binding of chimeric or humanized antibodies to murine C1q or FcγR as the contact sites for C1q in murine IgG2b and human IgG1 are different (69), and binding of human Fc to murine FcR is not optimal. Moreover, xenotransplantation studies suffer from additional drawbacks. For example, the impact of murine microenvironment on the expression levels of CD20 molecules or complement regulatory proteins is unknown. What is the influence of human MHC class I molecules on NK cells activity in mice? Is there a role for damage-associated molecular patterns (DAMPs) in recognition of human cells? These studies do not allow to measure distant vaccinal effects due to a lack of adaptive immunity. The last variable can be eliminated by using mouse anti-CD20 mAbs, but murine CD20 lacks typical redistribution motifs, which are present in the human antigen (70). Therefore, murine cells do not respond to mAb ligation in the same way as human. Studies in animals transplanted with murine tumor cells transfected with human CD20 gene and treated with rituximab suffer from the same species-compatibility problem (human Abs may not interact efficiently with mouse effector cells) and the results are confounded by the possibility of inducing an immune response against xenogeneic CD20 antigen. All these limitations do not invalidate the results obtained in *in vitro* or *in vivo* studies but need to be considered during interpretation of experimental data.

Another important issue is that tumor development is a long-term process associated with suppression of effector mechanisms of the immune system. Moreover, rituximab is most effective in combination with chemotherapeutics that exert immunosuppressive effects and lead to development of leucopenia. All these effects are likely to affect mechanisms associated with cell-mediated effector functions to a higher degree than activation of the complement cascade.

6.1. Complement-dependent cytotoxicity

CDC is an innate effector mechanism that participates in elimination of extracellular microorganisms or infected cells. It can be triggered in a spontaneous, so called alternative pathway or following recognition of target cells by opsonins such as lectins or antibodies. The first step of immunoglobulin-mediated i.e. classical pathway of complement activation is binding of C1q component to Fc portions of antigen-bound IgG or IgM antibodies, which triggers a cascade of proteolytic events leading to formation of a membrane-attack complex (MAC, C5b-9) (71). Activation of the complement cascade may also trigger several immune-mediated mechanisms in addition to direct cytotoxicity. Byproducts of the complement activation, including C1q, C3b, iC3b, and C4b are effective opsonins that facilitate removal of immune complexes and dead tumor cell debris by phagocytic cells. Opsonizing C3b molecules that bind to complement receptors (CR) strongly enhance FcγR-mediated phagocytosis of IgG-opsonized cells by macrophages and neutrophils (72). Small complement fragments released during the activation cascade (C3a, C4a and C5a), collectively referred to as anaphylatoxins are potent chemoattractants for leukocytes, also capable of triggering basophil and mast cell degranulation, increasing vascular permeability or smooth muscle constriction (73). Moreover, C5a can increase expression of FcγRIII and suppress expression of inhibitory FcγRIIb in macrophages (see below) (74).

6.1.1. CDC - *in vitro* studies

The degree of complement activation depends on several factors that include a tumor subtype, CD20 expression level, the presence of complement regulatory proteins (CRP), or the class or type of antibody (Figure 4). Type I anti-CD20 mAbs can activate the classical pathway of complement cascade and a number of observations indicate that CDC participates in elimination of normal and malignant B cells *in vitro* (75-80). This is most straightforwardly shown in studies with primary and established B cell lines that undergo rapid lysis when co-cultured with type I mAbs and complement components (usually a serum). Follicular lymphoma (FL) and mantle cell lymphoma (MCL) cells are killed more effectively than cells of diffuse large B-cell lymphoma (DLBCL), or B-cell chronic lymphocytic leukemia (B-CLL) (79). A sigmoidal correlation exists between CD20 levels and rituximab-mediated killing via CDC, but not ADCC (80). Similarly, other studies indicate that the potency of CDC in anti-CD20 mAb therapy correlates to some extent with CD20 expression levels (77-78).

6.1.2. CDC - *in vivo* studies

Depletion of complement components by cobra venom factor (CVF) significantly reduces antitumor activity of anti-CD20 mAb in severe combined immunodeficient (SCID) mice (62, 81-82). Similarly, studies with C1q-deficient mice revealed that in syngeneic mouse models (murine cells stably transfected with a human CD20 gene) activation of the classical complement pathway is fundamental for elicitation of therapeutic activity of anti-CD20 mAbs, whereas the activity of NK

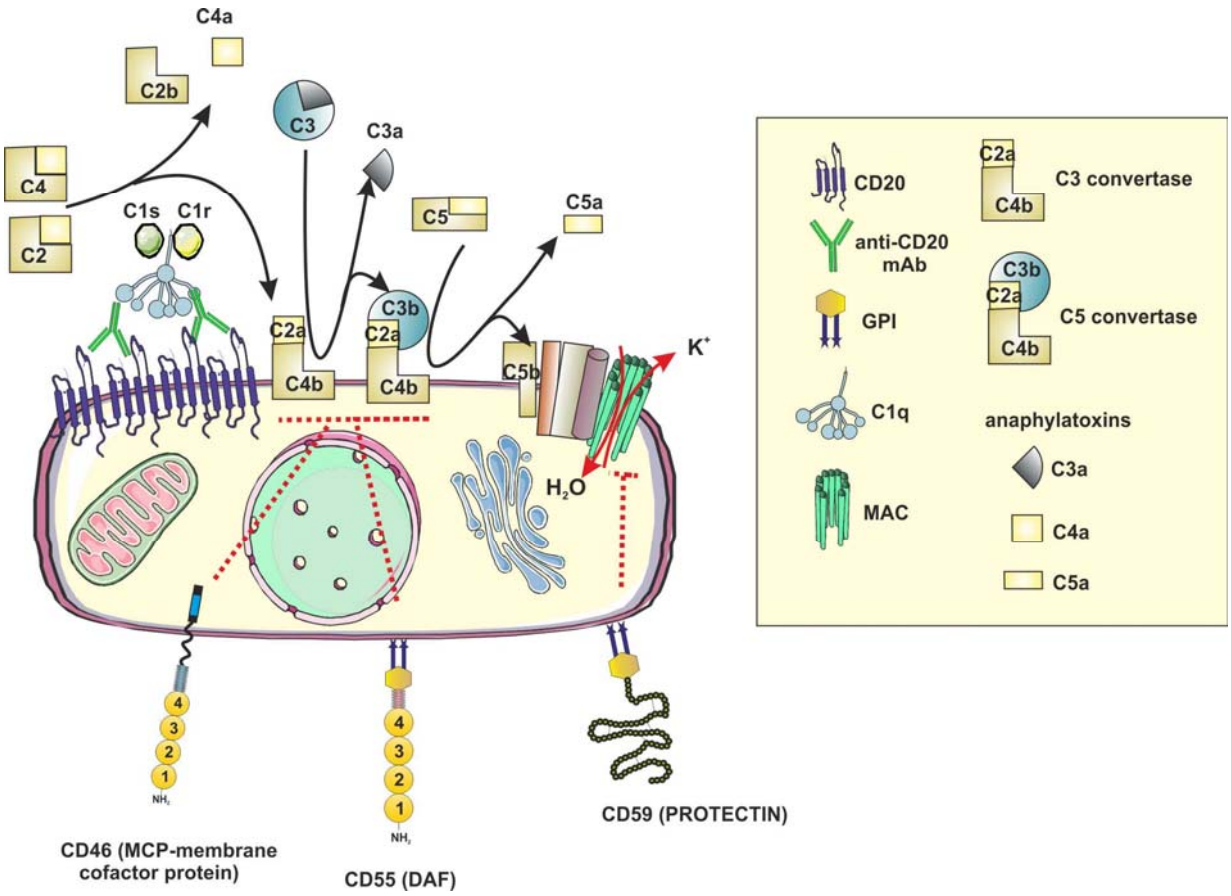

Figure 4. Activation of complement cascade by anti-CD20 mAbs.

cell, neutrophils, macrophages or T cells is dispensable (83-84). Studies in cynomolgus monkeys revealed that administration of rituximab induces a rapid (within 2 minutes) C3b(i) deposition in the plasma membrane of B cells that co-localizes with rituximab (85), and that the ability to eradicate CD20-positive cells with IgG1 mAbs was completely lost by the exchange of Fc fragments into IgG4, which lack complement-binding activity (86). Similarly, infusion of rituximab leads to deposition of C3b(i) in the plasma membranes of normal B cells (87).

Clinical observations indirectly confirm the involvement of complement activation in rituximab-treated patients. Administration of rituximab to CLL patients is associated with a profound loss of complement activity (87) or in a decrease in C2, C3 and C4 concentrations (87-88), which most likely results from complement consumption. Likewise, administration of rituximab increases plasma concentrations of complement activation products C3b/c and C4b/c in NHL patients (89). Moreover, treatment with rituximab leads to selection of CD59-positive cells in some patients with B-CLL (90), a finding also supported by similar observations in SCID mice (82).

At odds with these observations Ushida *et al.* reported that in mice deficient in C1q ($C1q^{-/-}$), C3 or C4 complement components anti-CD20 mAbs effectively deplete normal B cells (91). Also, a recent study revealed that rituximab-mediated depletion of B cells in human CD20 transgenic mice was comparable in wild-type and $C1q^{-/-}$ and $C3^{-/-}$ mice (92). In another study performed in BUB mice, which have exceptionally potent complement hemolytic activity, antitumor effects of genetically modified anti-CD20 mAbs with at least 30-fold reduced affinity for C1q were unaffected (93).

Also clinical studies provide ambiguous results on the role of complement in the antitumor efficacy of anti-CD20 mAbs. While C1qA polymorphism associated with low C1q concentrations correlates with prolonged response to rituximab in patients with FL (94), other studies indicate that administration of a fresh frozen plasma, which is a source of complement, improves antitumor activity of this antibody in patients with B-CLL (95). A recent study involving real-time imaging technique of living cells obtained directly from rituximab-treated patients revealed a close relationship between CDC susceptibility and clinical response to rituximab-containing chemotherapy (96). CDC susceptibility in this study was also significantly correlated with the level of CD20 expression.

Complement activation can be responsible for infusion-related side effects, which are associated with a substantial increase in blood components of activated

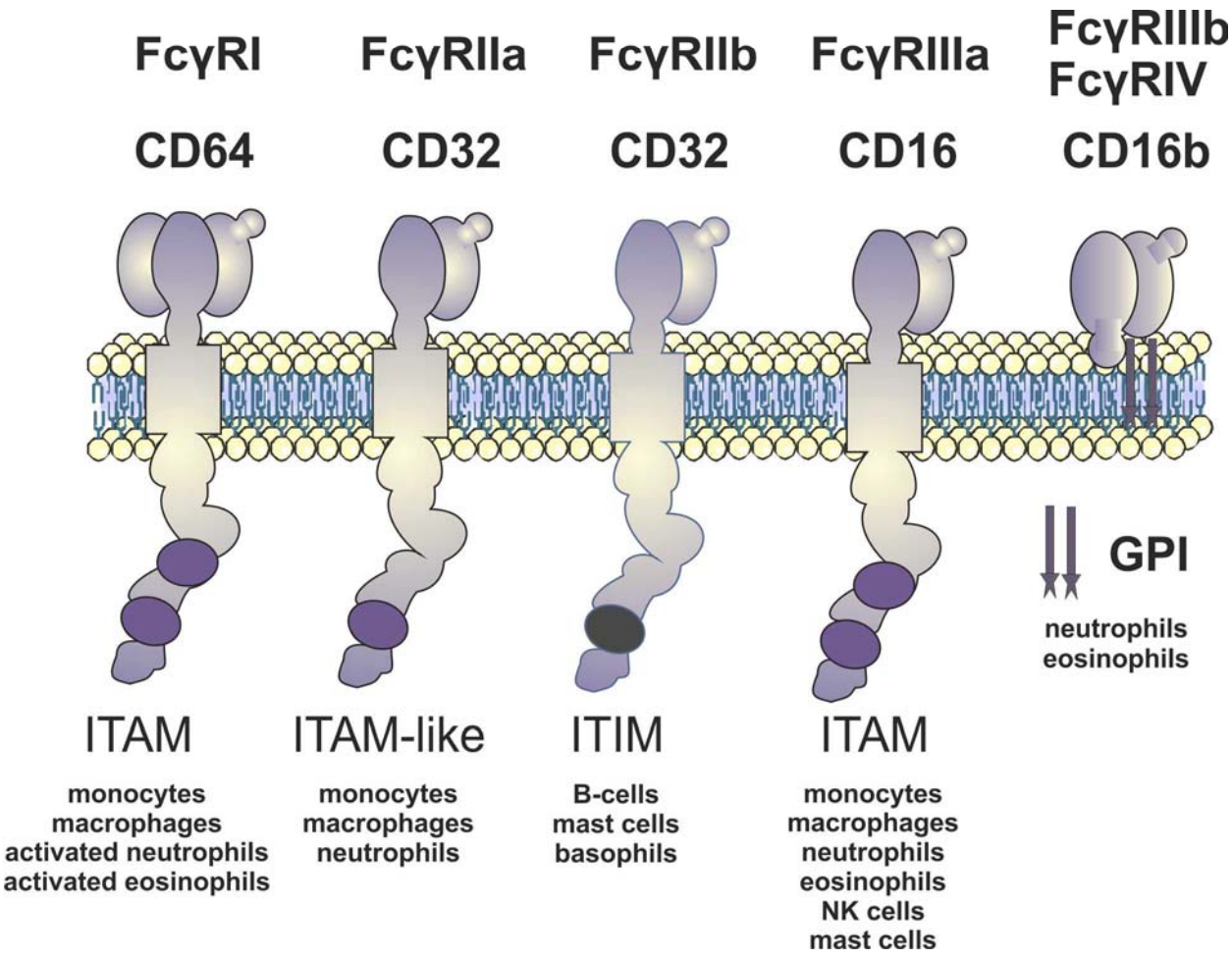

Figure 5. Activation of antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis by anti-CD20 mAbs.

complement cascade, mainly C3b and C4b (89). Additionally, some observations indicate that complement activation may even have detrimental effects in the setting of anti-CD20 mAb treatment. For example, C3 has been shown to directly inhibit the activity of NK cells and C3b components opsonizing tumor cells can inhibit ADCC mediated by these cells (97-98). Moreover, iC3b components attached to apoptotic cells favor development of immune tolerance-inducing dendritic cells (99). Phagocytic cells may participate in "shaving" reaction, which leads to removal of immune complexes together with rituximab and CD20 molecules from circulating tumor cells (see below).

6.2. FcγR-mediated cytotoxicity

Activation of effector cells of the immune system seems to be the most universal mechanism of cytotoxic effects as it is induced by both type I and type II anti-CD20 mAbs. CD20 binding by these antibodies on target tumor cells activates monocytes, macrophages, neutrophils, NK cells or γδ T cells via receptors for the constant fragment of IgG (FcγR). These receptors can be broadly divided into activating or inhibitory (100). Human activating receptors include FcγRI (CD64), FcγRIIA (CD32a), and FcγRIII (CD16). Activation of these receptors initiates signaling pathways through immunoreceptor tyrosine-based activation motifs (ITAM) found in cytoplasmic parts of FcγRIIA or in shared dimeric common γ chains that associate with FcγRI and FcγRIII. FcγRIIB (CD32b) is an inhibitory receptor that signals via immunoreceptor tyrosine-based inhibitory motifs (ITIM) (Figure 5).

Activation of effector cells via FcγR can result in various outcomes that depend on the type of FcγR engaged as well as the type and activation status of effector cells (Figure 6). Also the class and the type of anti-CD20 mAb, the presence of C3b or expression of MHC class I molecules in the target cells can influence the response of effector cells (101-103). Possible mechanisms of FcγR-mediated effects include: ADCC, immunophagocytosis, release of cytokines, proteases, reactive oxygen species (ROS) or crosslinking leading to induction of apoptosis (104). It still remains to be determined which cells and in which mechanisms participate in elimination of malignant B cells during anti-CD20 mAb treatment. These issues are difficult to address in a clinical setting. Studies in preclinical models are partly contradictory and it is formidable to interpret their results considering manifold

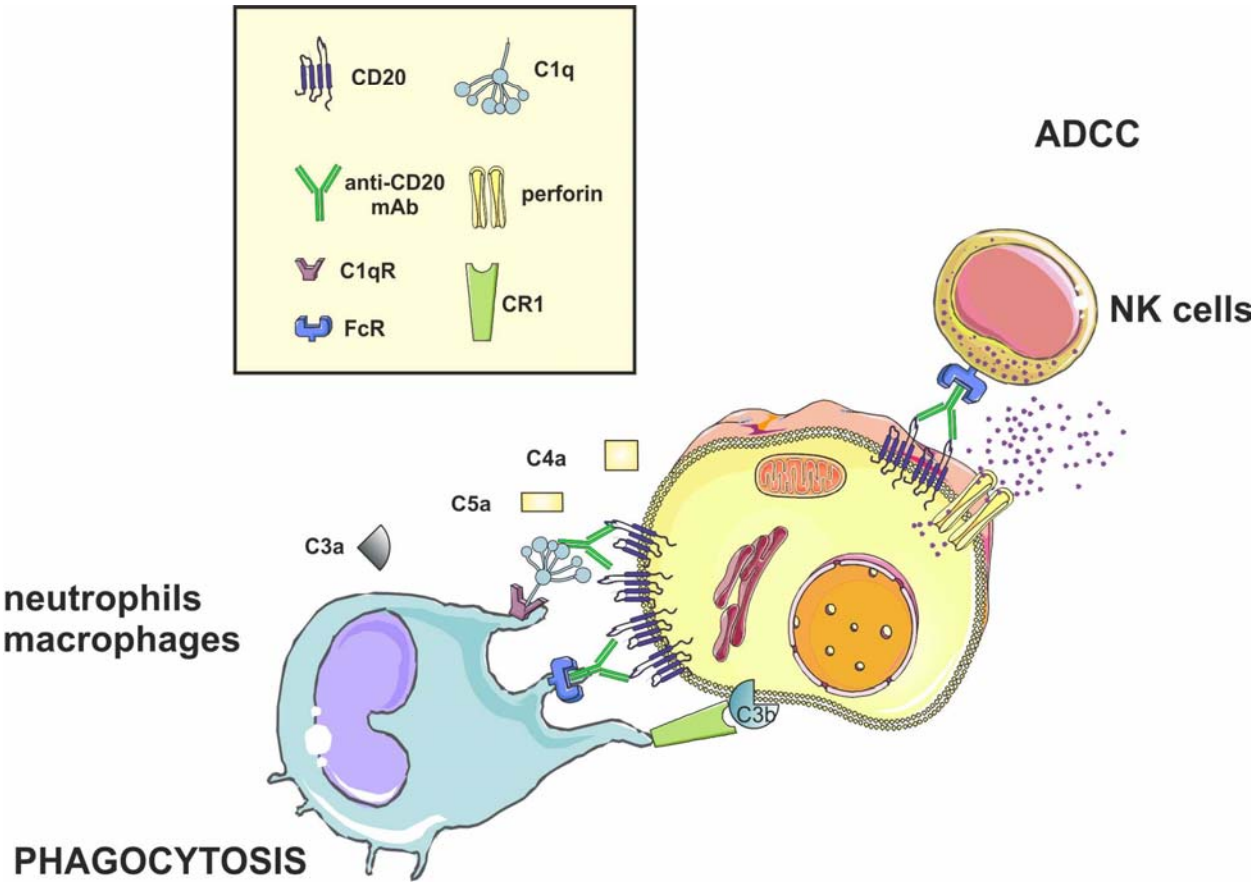

Figure 6. Direct induction of tumor cell death by anti-CD20 mAbs.

species incompatibilities between humanized (or human) antibodies and murine effector mechanisms (see above).

6.2.1. FcγR-mediated cytotoxicity - *in vitro* studies

Rituximab can induce ADCC of CD20-positive tumor cells by human peripheral blood mononuclear cells (PBMC) (105-106). Most of the ADCC activity of PBMC can be attributed to NK cells. Freshly isolated NK cells can kill up to 3 rituximab-coated target cells within 16 h. This number can be increased 2-fold by addition of IL-2 (107). Some studies indicate that peripheral blood-derived monocytes show *in vitro* anti-CD20 mAbs-mediated ADCC cytotoxicity towards malignant B-cells (108-109), but other do not confirm this, but indicate that only M-CSF-stimulated macrophages differentiated from $CD14^+$ monocytes can mediate phagocytosis of rituximab-coated B-CLL cells (110). Neutrophils and γδ T cells are another population of cells that can exert cytotoxic effects towards rituximab-opsonized tumor cells (111-112).

Many variables affect susceptibility or resistance of tumor cells to anti-CD20 mAb-mediated cytotoxicity. These are associated with the presence or expression of molecules that regulate the activity of effector cells. For example, expression of ULBP molecules, which serve as ligands for activating NKG2D receptors increases susceptibility of NHL cells to NK cell-mediated ADCC (113). On the other hand, a negative correlation with the expression of MHC class I molecules was found (113-114). Moreover, deposition of C3b on target cells impairs the interaction between the Fc fragment of rituximab and NK-cell receptor - FcγRIII (CD16), decreasing effector cell activation and ADCC (98). Also in mice C3 depletion enhanced NK-cell activation and improved the effectiveness of rituximab therapy (97).

6.2.2. FcγR-mediated cytotoxicity - *in vivo* studies

Unambiguous role of the cell-mediated mechanisms participating in elimination of tumor cells was revealed in experiments done with *FcRγ*$^{-/-}$ mice, that do not have common γ chains associating with FcγRI, FcγRIII and FcγRIV (a mouse ortholog of human FcγRIIIa). Antitumor effects of anti-CD20 mAbs are nearly completely abolished in these animals indicating that FcR-dependent mechanisms significantly contribute to *in vivo* activity of these antibodies (93, 115). This conclusion is supported by a growing number of clinical reports showing superior antitumor effects of rituximab in patients with NHL or Waldenström's macroglobulinemia having FcγRIIIa allelic variants that bind IgG1 with higher affinity (116-118). Of interest, the influence of the *FCGRIIIA* polymorphism is completely lost when rituximab is combined with chemotherapy (119-121). No association between FcγR polymorphic variation and response to treatment in patients

with CLL has been found (122). This is especially intriguing considering that induction of CDC requires about 10-fold more Abs deposited on the cell surface than does ADCC (123). Therefore, at low CD20 expression levels, which is typical for CLL, ADCC mechanisms might be dominant. Similarly, an allelic variant of FcγRIIA that binds IgG with higher affinity has been independently associated with better response rates in patients with FL (117). Treatment with anti-CD20 mAbs of mice deficient in inhibitory FcγRIIB resulted in better antitumor responses (93, 115), but it seems that polymorphism of genes encoding an inhibitory FcγRIIB does not affect response to rituximab in patients with FL (124).

In contrast to complement-mediated lysis the efficiency of macrophage-mediated phagocytosis is not affected by CD20 expression levels (110). Although the polymorphism of CD16 seems to affect ADCC of rituximab-coated targets by NK cells it does not affect phagocytosis by macrophages (110). Several mechanistic studies in mice revealed that normal and malignant B-cell depletion with anti-CD20 mAbs requires monocyte/macrophage FcγR expression (91, 93, 125). Clinical studies support the positive correlation between tumor-associated macrophages (TAMs) and response to rituximab (126-127).

Neutrophils are another leukocyte population that can contribute to the antitumor effects of anti-CD20 mAbs. Resting neutrophils exhibit low levels of FcγRIIa and FcγRIIIb. The latter is expressed exclusively by these cells and is involved in immunophagocytosis rather than ADCC. *In vitro* studies revealed that neutrophils participate in ADCC by using FcγRI, especially upon activation with G-CSF or IFN-γ and to a lesser degree by using FcγRIIa. However, these cells are far less potent in killing tumor cells as compared with NK cells (111, 128). Experiments in mice revealed that anti-lymphoma effects of rituximab are significantly reduced in neutrophil-depleted SCID mice and completely abrogated in neutrophil- and NK cells-depleted animals (129). However, murine, but not human, neutrophils mainly express FcγRIV, which can bind human IgG1, and clinical observations indicate that there is no association between polymorphism of FcγRIIIb and response to rituximab (130).

6.3. Direct cell killing

Based on morphologic and biochemical criteria several types of programmed cell death processes have been identified. Apoptosis - a historically classical programmed cell death - is executed by either external or internal pathway (131-132). The former is initiated by stimulation of death receptors, such as Fas (CD95) or TRAIL-R, and results in caspase 8 or 10 activation followed by proteolytic activation of caspase 3. During the intrinsic pathway BH3-only members of the Bcl-2 family regulate the release of cytochrome c from mitochondria. Cytochrome c together with Apaf-1 activate caspase 9, which triggers activation of downstream caspases 3 and 7. Caspase 3 degrades poly-(ADP-ribose)-polymerase (PARP – one of the enzymes repairing DNA injuries), activates phospholipase C, and leads to proteolysis of DNaze inhibitor, what results in DNA fragmentation. A type 2 or autophagic cell death is characterized by a massive vacuolization of the cytoplasm. Autophagic cytoplasmic degradation requires the formation of a double-membrane structure called the autophagosome, which sequesters cytoplasmic components as well as organelles and traffics them to the lysosomes (132). A third type of cell death is necrosis. Although it has long been described as a passive, accidental and unorganized way to die, recent evidence suggests that necrotic cell death can be actively propagated as part of signal transduction pathways (133).

6.3.1. Direct cell killing - *in vitro* studies

Anti-CD20 mAbs can induce death of malignant B cells (Figure 7) (51, 134). The mechanism of direct cytotoxic effects varies depending on the type of mAbs used. Type II mAbs, such as tositumomab more effectively induce direct killing of most B-cell tumor cell lines (52). In most studies crosslinking of rituximab or other type I mAbs with secondary antibodies is necessary to elicit apoptotic cell death. It is achieved by binding two molecules of rituximab by an antibody that recognizes their Fc fragments (crosslinking) (135), using a homodimerized rituximab (136), modified self-associating antibodies (137), or a dextran polymer of rituximab (138). However, the existence of *in vivo* crosslinking is controversial since the production of antibodies against rituximab is not observed in patients (139). It is hypothesized that crosslinking can be achieved by FcγR-bearing cells (135), but such possibility was recently ruled out (140). Although the existence of crosslinking has not been formally proven several authors report induction of caspase activation after rituximab administration *in vivo* or in patients with B-CLL (141-142).

After cross-linking rituximab can activate apoptosis via both external (143) and internal (41, 141, 144) pathways, as well as caspase-independent, in which there is no PARP degradation or chromatin condensation (145-146).

The involvement of death receptor-mediated apoptosis in anti-CD20 mAb-mediated cytotoxicity was hypothesized after observing that these antibodies induce homotypic adhesion of tumor cells. Clustering of tumor cells could enable direct interactions between cell surface molecules participating in extrinsic pathways of cell death. Indeed, in cells incubated with anti-CD20 mAbs an increased expression of Fas is observed (53, 147). Blocking anti-FasL antibodies decrease the percentage of tumor cells undergoing apoptosis (53) and anti-Fas antibodies synergize with rituximab in killing of $CD20^+$ tumor cells (147). Similarly, improved *in vitro* as well as *in vivo* effects of rituximab were observed in combination with anti-TRAIL-R mAbs (mapatumumab or lexatumumab) or with recombinant human TRAIL (148-149).

In some studies rituximab was shown to directly induce cytotoxic effects towards tumor cells, without any crosslinking (135, 150). Intriguingly, this effect is observed in some but not all $CD20^+$ tumor cell lines (151). A recent study revealed that susceptibility to direct cytotoxicity correlates with the level of GM1 gangliosides (152), which

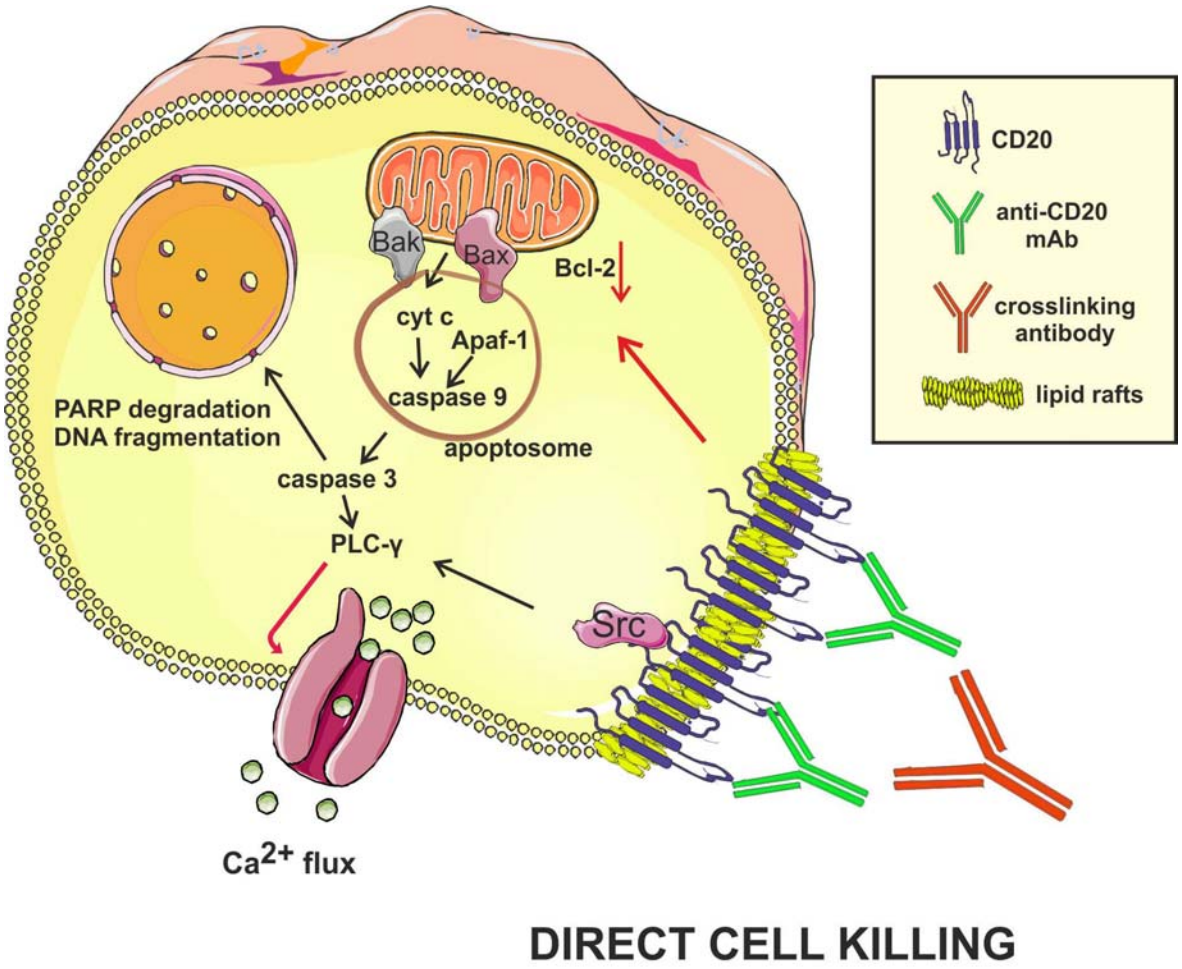

Figure 7. Structure and expression of human and mouse FcγRs.

are sialic acid-containing glycosphingolipids that participate in the formation and stabilization of lipid rafts. Based on these observations it is possible that type I antibodies can trigger death of $CD20^+$ cells only when CD20 molecules can be translocated to lipid rafts containing high levels of GM1 gangliosides or after crosslinking. In contrast, type II antibodies readily induce cytotoxic effects without the need for raft translocation. The mechanisms of these effects remain to be elucidated.

Cell death induced by type II mAbs is not a classic apoptosis. It does not lead to caspase processing or DNA fragmentation, seems to be independent of Bcl-2 activity, and does not involve autophagy (153-154). Instead, these antibodies induce homotypic adhesion of tumor cells associated with rapid and sustained actin redistribution that leads to structural changes in lysosomes that swell and release their contents, including cathepsin B, into the cytoplasm precipitating loss of plasma membrane integrity and cell death (154).

6.3.2. Direct cell killing - *in vivo* studies

Only few studies show direct induction of apoptosis by anti-CD20 mAbs. In xenografted human lymphoma model rituximab treatment induces activation of caspases (142). Activation of caspase-3, caspase-9 and PARP cleavage have also been observed in some CLL patients treated with rituximab indicating that direct apoptosis might be involved in elimination of tumor cells (141). However, B cells transfected with Bcl-2 gene that are resistant to apoptosis-inducing agents are efficiently eliminated from mice (92). Clinical studies revealed that patients with CLL not responding to rituximab treatment have an increased ratio of anti-apoptotic Mcl-1 to pro-apoptotic Bax ratio, although the pretreatment levels of other anti-apoptotic proteins, such as Bcl-2 or XIAP had no influence on the treatment efficacy (90). Preclinical studies revealed that antisense Bcl-2 oligonucleotides or AT-101, a BH3 domain (Bcl-2 homology domain 3) mimetic, strongly potentiate rituximab-mediated effects in SCID mice transplanted with human lymphoma xenografts (155-156). Promising and beneficial effects of combination of rituximab with sodium oblimersen, a Bcl-2 antisense oligonucleotide, in patients with indolent NHL have also been observed (157).

Type II antibodies retain their full antitumor activity in NK cell-depleted or NK cell-deficient (Beige)

mice (62). $F(ab')_2$ fragments from these antibodies retained significant *in vivo* activity and both B1 and its $F(ab')_2$ fragments effectively induced apoptosis of $CD20^+$ lymphoma cells indicating that direct cytotoxic effects rather than CDC, ADCC or phagocytosis are responsible for their tumoricidal activity (62).

6.4. Vaccinal effects

Administration of anti-CD20 mAbs to patients with B-cell tumors most likely triggers all of the effector mechanisms at the same time. It should be emphasized that CDC, ADCC or induction of cell death are not interdependent or mutually exclusive but most likely interact and cooperate in elimination of malignant B cells. Delayed responses observed in some patients treated with rituximab led to suggestions that anti-CD20 mAbs might also favor development of adaptive immune responses. Tumor cells damage by complement or during ADCC might be associated with an increased availability of fragments of tumor cells opsonized by antibodies and/or complement fragments (158). Moreover, rituximab-binding to lymphoma cells can induce expression of CCL3 and CCL4 chemokines (81) that together with complement activation products (C3a, C5a) could participate in recruitment and activation of antigen presenting cells (APC) such as dendritic cells or macrophages. In transplantation, for example, C3a and C5a receptors on donor APC are critical for triggering T-cell-mediated rejection (159-160). Chemoattracted and activated APC might then effectively capture damaged tumor cells, process engulfed proteins and present tumor-associated antigens in association with major histocompatibility complex (MHC) molecules to T cells. *In vitro* studies confirmed that lymphoma cells undergoing rituximab-induced apoptosis can be taken up by immature dendritic cells, induce their maturation and promote cross-presentation to activate $CD8^+$ cytotoxic T cells (161). Although anti-CD20 antibody treatment or dendritic cell vaccination alone showed minimal antitumor effects in mice, the combined treatment resulted in significant long-term survival (162). A small exploratory clinical study revealed that in 4 out of 5 patients with follicular lymphoma rituximab treatment is capable of inducing idiotype-specific T-cell response (163). Also, a booster effect, i.e. a favorable and quick response to rituximab after the second infusion might indirectly confirm the possible existence of rituximab-induced "vaccinal" effects (164).

7. FACTORS AFFECTING THE ACTIVITY OF ANTI-CD20 MABS

Although rituximab is a critical monoclonal antibody in the treatment of CD20-positive B-cell malignancies the resistance to this treatment has been a considerable clinical problem. Unfortunately, a significant percentage (50%) of patients who initially respond to rituximab-based first line therapies eventually relapse (139, 165). Moreover, although more than 95% of patients with NHL express surface CD20 there are patients who demonstrate intrinsic resistance to initial therapy. There is mounting evidence supporting the existence of multiple mechanisms of antitumor rituximab action, but the mechanisms of rituximab resistance are still poorly understood.

7.1. The expression of CD20

CD20 expression is quite heterogeneous in various lymphoma subtypes. Typically, CLL have a lower (dim) CD20 expression and this seems to be responsible for the lower response rates to rituximab treatment as compared with FL and MCL. Several studies have addressed whether the level of CD20 expression correlates with efficacy of rituximab and may be used to predict progression of disease and response to treatment. The results are to some extent conflicting. In some *in vitro* studies with cells derived from patients suffering from various B-cell malignancies a positive correlation between CD20 levels and rituximab sensitivity (R-CDC) was found (77-78). However, these observations were not corroborated by other studies (79). An elegant study by van Meerten *et al.* has demonstrated a sigmoidal correlation between CD20 expression level and rituximab-mediated CDC but not ADCC (80). In this *in vitro* experimental model the level of CD20 expression was the only variable and it was clearly shown that reduced CD20 expression leads to impaired CDC. A direct correlation between R-CDC and the number of CD20 molecules in primary NHL cells was also found by Bellosillo *et al.* (77). It has been established that the minimum level of 5×10^4 CD20 molecules per cell is necessary for rituximab to induce CDC (77). It should be mentioned that MACs formation leads to rapid necrotic-type cell death only when multimers of MACs are assembled (166-167), which in turn depends on CD20 levels. Strategies that induce upregulation of CD20 expression may improve rituximab-mediated cell kill of low CD20-epxressing cells and provide a rationale for overcoming rituximab-resistance.

On the contrary, all processes that cause CD20 decrease, downregulation, changes in epitope structures, or in CD20 localization within cellular membrane could potentially impair antitumor activity of rituximab-based therapies and lead to rituximab resistance

7.1.1. Transcriptional regulation

It is still frequently taken for granted that CD20 expression levels in various B-cell tumors is relatively constant. However, accumulating evidence indicates that CD20 can be modulated at several levels, both transcriptional and posttranscriptional, and this can even lead to selection of antigen-loss variants of tumor cells. Several case or retrospective studies reported that CD20-negative relapses after rituximab treatment do occur (168-175). The prevalence and duration of CD20 loss are currently unknown. A number of mechanisms that account for modulation of CD20 levels have been proposed. Most likely their occurrence and significance varies depending on the type of malignancy. In CLL rituximab-mediated down-modulation of CD20 is associated with reduced levels of CD20 mRNA both *in vitro* (176) and *in vivo* (177) indicating that transcriptional regulation participates in this process. Indeed, a recent study with CLL cells indicated that Flt3

ligand (FL) activates signaling cascades that inhibit expression of PU.1, a transcription factor involved in the expression of *cd20* gene (178). Rituximab-mediated down-regulation of CD20 mRNA and protein levels in CLL was transient and did not lead to selection of antigen-loss variants (177).

Epigenetic mechanisms also play an emerging role in the regulation of CD20 levels. This was first reported in classic Hodgkin lymphomas that normally do not express CD20, but do so in response to a DNA demethylating agent 5-aza-deoxycytidine (5-aza-dC) (179). Downregulation of CD20 mRNA has been also observed in CD20-negative cells obtained from patients after relapse of rituximab-treated B-cell malignancies (175). Also in these cases 5-aza-dC restored CD20 mRNA expression, increased CD20 surface levels and sensitized tumor cells to rituximab-mediated ADCC (175). It is noteworthy that CpG islands are absent from the CD20 promoter region, located ~5 kb upstream of the transcription site, and CpG methylation of the CD20 promoter has not been observed in CD20-negative transformed cells (175, 180). Thus, it can be hypothesized that altered, epigenetically-regulated expression of transcription factors critical for CD20 gene expression may contribute to the aberrant CD20 transcriptional regulation.

Another epigenetic mechanism associated with modulation of CD20 expression is regulated by histone acetylation. Tomita *et al.* have established a CD20 negative lymphoma cell line (RRBL1) from a patient treated repeatedly with rituximab-containing chemotherapy. Decreased expression of CD20 has been observed at both protein and mRNA level and was completely reversed by trichostatin A, an epigenetic drug that modulates histone-acetylation status (181).

7.1.2. Posttranscriptional regulation

It is frequently underscored that CD20 molecules are not endocytosed from tumor cells after antibody binding. However, it was demonstrated that 1F5 (182-183) as well as rituximab (176, 184) can be internalized by malignant cells. Also stimulation of normal B cells through CD40 has been shown to down-regulate CD20 expression, which occurs by protein kinase C-dependent endocytosis (185). Internalization of CD20 can be also induced by lenalidomide (186). Recent studies indicate that only type I anti-CD20 mAbs induce CD20 internalization and that this mechanism, rather than shaving, is responsible for modulation of this antigen (92). It is uncanny that CD20 internalization has been overlooked so far. Detailed molecular mechanisms that are responsible for this process are urgently needed to design rational combination therapies allowing more effective management of B-cell tumors.

Even more enigmatic are mechanisms that might lead to shedding of CD20 molecules. The presence of soluble, circulating CD20 molecules has been demonstrated in patients with NHLs or CLL (187-189). Considering a tight association of tetraspanins with plasma membrane it is possible that CD20 molecules detected in the serum are released from B cells as exosomes or these are merely fragments of tumor cells released in response to damage induced by CDC or ADCC.

NHL cells chronically exposed to rituximab acquire a resistant phenotype associated with reduced surface CD20 levels and decreased sensitivity to rituximab-mediated CDC and ADCC (190). Microarray cDNA analysis revealed that rituximab-resistant cells reveal decreased surface CD20 levels and exhibit up-regulation of the components of the ubiquitin-proteasome system (190). Proteasome inhibition partially reversed rituximab resistance (190) indicating that CD20 might be a substrate of intracellular proteolytic degradation systems. Recent studies indicate that indeed CD20 can be ubiquitinated and proteasome inhibitors bi-modally regulate surface CD20 levels - short-term inhibition of proteasome activity up-regulates CD20 levels, while prolonged inhibition leads to autophagic degradation of these molecules (191).

A novel splice variant of mRNA that encodes a truncated, non-anchored CD20 protein (ΔCD20) selectively expressed in malignant, but not resting B cells was recently isolated from healthy donors (27). This protein apparently associates with intracellular domains of normal CD20 and its levels increase in rituximab-resistant cells. It is possible that association with ΔCD20 can modulate the levels of CD20 in the plasma membrane or may affect its translocation to lipid rafts.

Finally, conformational changes or altered reorganization of CD20 within lipid rafts can be associated with reduced binding of anti-CD20 antibodies to discontinuous epitopes formed within the larger or between the extracellular loops of CD20 (33, 192).

7.1.3. CD20 mutations

CD20 mutations have been detected in 11 (22%) out of 50 patients with non-Hodgkin's B-cell lymphomas treated with rituximab-containing therapies (193). Especially C-terminal deletions have been strongly associated with decline or disappearance of CD20, a short time to progression, and early relapse of disease. In DLBCL CD20 mutations were shown to be extremely rare before rituximab treatment indicating that they do not account for primary resistance (194-195). However, of the rare samples available after R-CHOP almost 20% had been CD20-negative and one contained *cd20* mutation (194). Another study in Japan reported two initially CD20-positve patients with relapsed DLBCL, who transformed into CD20-negative tumors harboring *cd20* mutations (175).

7.1.4. Other mechanisms

Treatment with anti-CD20 mAbs may saturate or exhaust effector mechanisms responsible for elimination of tumor cells. Rituximab-mediated ADCC of NK cells leads to downregulation of FcγRIIIa (196). Similar effects were observed with other mAbs, such as trastuzumab (197). Re-expression of FcγRIIIa may take as many as 24 h (198). Similarly, the complement system can be exhausted as a result

of rituximab treatment, and restoration of complement components concentrations can take even longer, from a few days up to several weeks (87). Saturation of effector mechanisms may enable alternative processing pathway referred to as "shaving" (199). In this reaction the complexes of antibodies and their targets are extracted from tumor cells in a process of trogocytosis, a process of plasma membrane exchange within immunological synapse that forms between the tumor and FcγR-bearing cell (64, 200-201). Trogocytosis leads to removal of CD20 molecules from tumor cells and selects for cells resistant to anti-CD20 mAbs (199). Fractionated small dose or subcutaneous dosing schedules limit exhaustion of effector mechanisms and may be more effective than standard intravenous bolus rituximab injections in CLL patients (199, 202-203).

7.2. CD20 independent mechanisms of resistance to anti-CD20 mAbs

A number of complement regulatory proteins (CRP) protect normal cells from CDC. Three of them are believed to be the most important: CD46 (MCP – membrane cofactor protein that binds to and serves as a cofactor in the cleavage of C3b and C4b), CD55 (DAF – decay accelerating factor, which accelerates inactivation of C3 and C5 convertases) and CD59 (which impairs MAC formation). These inhibitors are also present on tumor cells, and might facilitate their escape from complement attack (204). Indeed, expression of CD55 and CD59 limits rituximab-mediated CDC in B-cell tumors (79, 205).

Furthermore, tumor cells may become refractory to R-ADCC in mechanism involving resistance to enzymatic activity of granzymes and perforin. It has been demonstrated that expression of protease inhibitor 9 (inhibitor of granzyme B) is used by tumor cells to escape their elimination by effector cells of the immune system (206).

8. APPROACHES TO IMPROVE ANTITUMOR EFFICACY OF ANTI-CD20 MABS

8.1. Complement-dependent cytotoxicity

Antibodies blocking complement regulatory proteins, the use of phospholipase C that cleaves off a GPI anchor from CD55 and CD59, or siRNA that knocks-down CRP expression facilitate rituximab-mediated cytotoxicity (77, 207-210). Also in a xenograft *in vivo* models it was shown that antibodies blocking the activity of CD55 and CD59 (211) or a recombinant adenoviral fiber knob protein that cross-links CD46 molecules (212) enhance therapeutic effects of rituximab. Fludarabine, the nucleoside analogue clinically active against CLL and indolent NHL has been shown to act synergistically with rituximab and to down-regulate the membrane expression of CD55 without significantly altering CD20 levels (213).

Recently, extracellular protein kinases have been shown to regulate activation of the complement cascade. One such ectokinase is casein kinase 2 (CK-2), which phosphorylates serine residues at the N-terminus of C9 component, impairing its joining to C5b-8 and formation of the fully functional MAC (214). Inhibition of ecto-CK-2 activity may increase rituximab efficacy (215).

Another approach that exploits the ability of rituximab to trigger complement activation is based on administration of antibodies specific for C3b and iC3b, the cell-associated complement C3 cleavage products (216).

However, not all studies confirm the significant role played by CRP. For example, pretreatment levels of CD46, CD55 and CD59, or combinations of these proteins, did not predict clinical responses to rituximab in patients with FL (217). In B-CLL the expression levels of CD46, CD55 and CD59 did not correlate with R-CDC, but antibodies blocking CRPs increased lysis of tumor cells incubated with rituximab and complement (78).

8.2. Antibody-dependent cell-mediated cytotoxicity

Strategies to augment ADCC are usually directed at increasing the killing activity of effector cells. This is most easily achieved by activating the function of neutrophils, macrophages or NK cells with recombinant cytokines. A number of hematopoietic growth factors (G-CSF, GM-CSF, M-CSF), interleukins (IL-2, IL-12, IL-15, IL-21), chemokines (macrophage chemotactic protein-1), and interferons (IFN-α, IFN-γ) have been shown to potentiate R-ADCC *in vitro* (15, 109-111, 218-220). These studies were followed by clinical trials that indicated effectiveness of several approaches. For example, in a small study involving 15 patients with FL addition of G-CSF to a standard R-CHOP regimen resulted in 100% overall response rate with 12 patients achieving complete response (221). Combination of G-CSF with rituximab (no chemotherapy) also seems to prolong the duration of remission in patients with low-grade lymphomas (222). Similarly, GM-CSF enhances rituximab activity in patients with FL (223). Although in animal studies administration of IL-2 significantly potentiated anti-CD20 mAb-mediated antitumor effects (5), clinical studies did not show improved responses in patients concomitantly treated with rituximab and rhIL-2 (224). However, the clinical study included a rather small group of patients and no other factors such as FcγR polymorphism were evaluated that might influence the therapeutic outcome of the combination treatment. Additionally, recent clinical observations indicate that also high serum soluble interleukin-2 receptor levels might be associated with inferior prognosis and might affect responses to rituximab (225). Combination of rituximab with IL-12, a cytokine that strongly stimulates cell-mediated immunity (226), has so far been disappointing in clinical trials (227). Similarly, combination with IFN-α-2a has not lived up to its expectations (228).

Next to recombinant cytokines also other immunomodulatory drugs have been used to improve effector functions of immune cells in combination with rituximab. These include toll-like receptor 9 (TLR9) agonists - CpG oligonucleotides (229), β-1,3-glucan, which binds to the lectin domain of the leukocyte complement receptor CR3, and facilitates binding to iC3b thereby triggering cytotoxicity of iC3b-coated tumor cells (230), or lenalidomide, which stimulates cytotoxicity of NK cells,

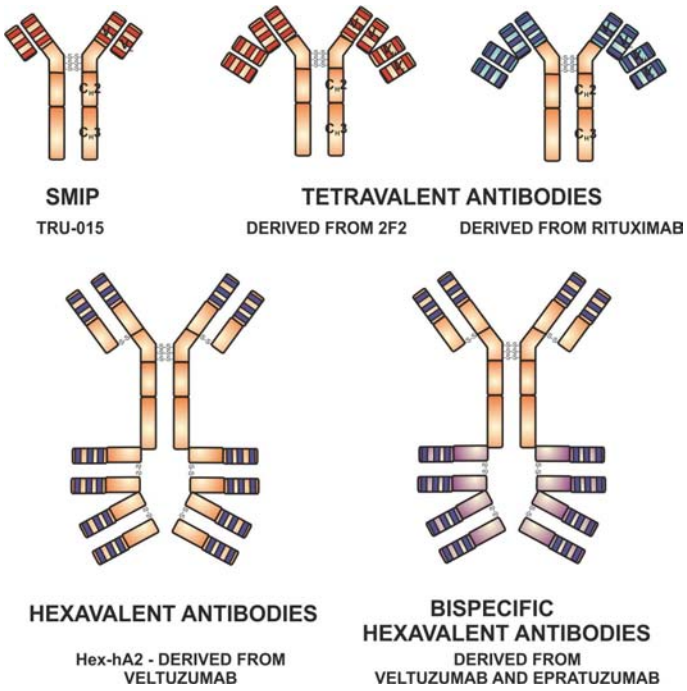

Figure 8. Novel anti-CD20 mAbs.

increases production of IL-2, and inhibits angiogenesis (231).

8.3. Combination therapies

There are multiple reasons that substantiate combination of anti-CD20 mAbs with chemotherapeutics. These include independent mechanism of antitumor action, non-overlapping toxicities, antibody-mediated sensitization to cytostatic/cytotoxic effects of chemotherapeutics. Rituximab exhibits synergistic activity with many chemotherapeutics, e.g. antimetabolites (metotrexat (232), fludarabine (233)), anthracyclines (doxorubicin (234)), taxoids (paclitaxel (235)), lignans (etoposid (236)) and alkylating agents (cyclophosphamide (234), chlorambucil (237), cisplatin (238)). To achieve even greater effectiveness the above-mentioned drugs are joined into schemes. In clinical trials the addition of rituximab to standard schemes, like CHOP (cyclophosphamide, hydroxy-daunorubicin, Oncovin, prednisone) (239), EPOCH (etoposide, prednisone, Oncovin, cyclophosphamide, hydroxydaunorubicin) (240) or CVP (cyclophosphamide, vincristine, prednisone) (241) increased the treatment effectiveness, and the observed synergism allowed to use lower doses evoking less side effects. Cytostatics impair cell divisions and cause many sublethal injuries inducing apoptosis, and rituximab by decreasing Bcl-2 level sensitizes cells to their activity. As could be presumed it does not happen is a one peculiar mechanism, but as a result of several overlapping and complementary intracellular pathways (235, 242-245).

Moreover, rituximab acts synergistically not only with standard chemotherapeutics, but also with new drugs like bortezomib in patients with various B-cell malignancies (246-248), thalidomide (249), lenalidomide, a thalidomide-derivative immunomodulator (250), everolimus (mTOR inhibitor) (251), tenespimycin (Hsp90 inhibitor) (252) or anti-malarial artesunate (253). Also new antibodies are added to rituximab therapy, e.g. galiximab (anti-CD80) (254), alemtuzumab (anti-CD52) (255), bevacizumab (anti-VEGF) (256) or epratuzumab (anti-CD22) (257). By joining calicheamicin with rituximab an immunotoxin was obtained, which more effectively evokes apoptosis, simultaneously not impairing CDC or ADCC effectiveness (258).

9. CONCLUSIONS

A number of variables affecting the interactions between target and effector cells seems to influence the efficacy of mAbs. These include mAbs concentration, their affinity for target antigen, target antigen density, epitope specificity for target antigen, type of effector cells as well as mAbs affinity for FcR. Modifications of anti-CD20 mAbs fine-tuning all these variables are therefore expected to increase their therapeutic activity. Several studies have addressed this possibility. Oligonucleotide-directed mutagenesis of immunoglobulin genes led to design of anti-CD20 mAbs with higher affinity for CD20, FcγRIII or both, which were more potent in triggering ADCC against tumor cells (106). Removal of fucose from anti-CD20 IgG1 antibodies potentiates NK cell-mediated ADCC against tumor cells by increasing antibody affinity for FcγR (259). A growing number of additional anti-CD20 antibodies or anti-CD20-targeted therapeutics are being developed in pre-clinical setting (Figure 8). These include rituximab triple mutant with extremely potent apoptosis-inducing activity (260), authophilic (self-associating) DXL625

antibody specifically designed to trigger enhanced apoptosis (261), hexavalent antibodies (Hex-hA2), which comprise 6 Fab and one Fc fragment (262), tetravalent antibodies (DiMcAb) derived from two anti-CD20 mAbs (rituximab and 2F2) (263), bispecific anti-CD20/CD22 antibodies derived from veltuzumab and epratuzumab (anti-CD22 mAb) (264), or even engineered T cells expressing CD20-specific chimeric T-cell receptors (TCR) (265).

It is difficult to reconcile many discrepant observations addressing engagement of effector mechanisms of anti-CD20 mAbs described in this review. The obvious disparities in results of experimental and clinical studies arise from different numbers of target cells (low numbers of transplanted tumor cells vs. larger numbers in long-term established tumors in patients), CD20 expression levels, and the fact that malignant B cells are usually heavily passaged before transplantation. Maintenance of tumor cells is media containing heat-inactivated serum results in down-regulation of CRPs thereby increasing their susceptibility to CDC. Another possibility is that transplanted malignant B cells frequently localize to different body compartments.

Identification of the mechanisms of cytotoxic effects of anti-CD20 mAbs is necessary to establish assessable molecular and genetic predictive factors that can be used to plan tailored therapeutic approaches in individual patients. This would mean that mAb therapy of lymphoma has entered a new age.

10. ACKNOWLEDGEMENTS

Supported by grants 1M19/N from the Medical University of Warsaw, N N402 352938 from Polish Ministry of Science and the European Union within European Regional Development Fund through Innovative Economy grant POIG.01.01.02-00-008/08. None of these organizations had any influence on neither the course of the studies nor on the preparation of the manuscript. J.G. and J.B. are recipients of the Mistrz Award from the Foundation for Polish Science. M.W. and J.B. are recipients of the START Award from the Foundation for Polish Science. J.G. and M.W. are members of TEAM Programme co-financed by the Foundation for Polish Science and the EU European Regional Development Fund. E.G-M. was recipient of "Mazowieckie Stypendium Doktoranckie" from the EU structural funds. Figures were produced using Servier Medical Art (www.servier.com) for which the authors would like to acknowledge Servier.

11. REFERENCES

1. Kohler G. and C. Milstein: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495-7 (1975)

2. Tyden G., H. Genberg, J. Tollemar, H. Ekberg, N. H. Persson, G. Tufveson, J. Wadstrom, M. Gabel and L. Mjornstedt: A randomized, doubleblind, placebo-controlled, study of single-dose rituximab as induction in renal transplantation. *Transplantation* 87, 1325-9 (2009)

3. Stasi R.: Rituximab in autoimmune hematologic diseases: not just a matter of B cells. *Semin Hematol* 47, 170-9 (2010)

4. Mo C., B. Vire and A. Wiestner: Rituximab: therapeutic benefit! Vitamin R? *Semin Hematol* 47, 105-6 (2010)

5. Hooijberg E., J. J. Sein, P. C. van den Berk, A. A. Hart, M. A. van der Valk, W. M. Kast, C. J. Melief and A. Hekman: Eradication of large human B cell tumors in nude mice with unconjugated CD20 monoclonal antibodies and interleukin 2. *Cancer Re*, 55, 2627-34 (1995)

6. Bindon C. I, G. Hale, M. Bruggemann and H. Waldmann: Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *J Exp Med* 168, 127-42 (1988)

7. Bubien J. K., L. J. Zhou, P. D. Bell, R. A. Frizzell and T. F. Tedder: Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. *J Cell Biol* 121, 1121-32 (1993)

8. Polyak M. J. and J. P. Deans: Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure. *Blood* 99, 3256-62 (2002)

9. van Meerten T. and A. Hagenbeek: CD20-targeted therapy: the next generation of antibodies. *Semin Hematol* 47, 199-210 (2010)

10. Goldenberg D. M., F. Morschhauser and W. A. Wegener: Veltuzumab (humanized anti-CD20 monoclonal antibody): characterization, current clinical results, and future prospects. *Leuk Lymphoma* (2010)

11. Zhang B.: Ofatumumab. *MAbs* 1, 326-31 (2009)

12. Oflazoglu E. and L. P. Audoly: Evolution of anti-CD20 monoclonal antibody therapeutics in oncology. *MAbs* 2, 14-9 (2010)

13. Hayden-Ledbetter M. S., C. G. Cerveny, E. Espling, W. A. Brady, L. S. Grosmaire, P. Tan, R. Bader, S. Slater, C. A. Nilsson, D. S. Barone, A. Simon, C. Bradley, P. A. Thompson, A. F. Wahl and J. A. Ledbetter: CD20-directed small modular immunopharmaceutical, TRU-015, depletes normal and malignant B cells. *Clin Cancer Res* 15, 2739-46 (2009)

14. Pawluczkowycz A. W., F. J. Beurskens, P. V. Beum, M. A. Lindorfer, J. G. van de Winkel, P. W. Parren and R. P. Taylor: 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* 183, 749-58 (2009)

15. Treon S. P., L. M. Pilarski, A. R. Belch, A. Kelliher, F. I. Preffer, Y. Shima, C. S. Mitsiades, N. S. Mitsiades, A. J. Szczepek, L. Ellman, D. Harmon, M. L. Grossbard and K. C. Anderson: CD20-directed serotherapy in patients with multiple myeloma: biologic considerations and therapeutic applications. *J Immunother* 25, 72-81 (2002)

16. Anderson K. C., M. P. Bates, B. L. Slaughenhoupt, G. S. Pinkus, S. F. Schlossman and L. M. Nadler: Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. *Blood* 63, 1424-33 (1984)

17. Tedder T. F. and S. F. Schlossman: Phosphorylation of the B1 (CD20) molecule by normal and malignant human B lymphocytes. *J Biol Chem* 263, 10009-15 (1988)

18. Stashenko P., L. M. Nadler, R. Hardy and S. F. Schlossman: Characterization of a human B lymphocyte-specific antigen. *J Immunol* 125, 1678-85 (1980)

19. Sun T., A. Akalin, M. Rodacker and T. Braun: CD20 positive T cell lymphoma: is it a real entity? *J Clin Pathol* 57, 442-4 (2004)

20. Algino K. M., R. W. Thomason, D. E. King, M. M. Montiel and F. E. Craig: CD20 (pan-B cell antigen) expression on bone marrow-derived T cells. *Am J Clin Pathol* 106, 78-81 (1996)

21. Quintanilla-Martinez L, F. Preffer, D. Rubin, J. A. Ferry and N. L. Harris: CD20+ T-cell lymphoma. Neoplastic transformation of a normal T-cell subset. *Am J Clin Pathol* 102, 483-9 (1994)

22. Fang D., T. K. Nguyen, K. Leishear, R. Finko, A. N. Kulp, S. Hotz, P. A. Van Belle, X. Xu, D. E. Elder and M. Herlyn: A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 65, 9328-37 (2005)

23. Parolini D., M. Meregalli, M. Belicchi, P. Razini, R. Lopa, B. Del Carlo, A. Farini, S. Maciotta, N. Bresolin, L. Porretti, M. Pellegrino and Y. Torrente: CD20-related signaling pathway is differently activated in normal and dystrophic circulating CD133(+) stem cells. *Cell Mol Life Sci* 66, 697-710 (2009)

24. Adra C.N., J. M. Lelias, H. Kobayashi, M. Kaghad, P. Morrison, J. D. Rowley and B. Lim: Cloning of the cDNA for a hematopoietic cell-specific protein related to CD20 and the beta subunit of the high-affinity IgE receptor: evidence for a family of proteins with four membrane-spanning regions. *Proc Natl Acad Sci U S A* 91, 10178-82 (1994)

25. Tedder T. F., M. Streuli, S. F. Schlossman and H. Saito: Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes. *Proc Natl Acad Sci U S A* 85, 208-12 (1988)

26. Tedder T. F., G. Klejman, C. M. Disteche, D. A. Adler, S. F. Schlossman and H. Saito: Cloning of a complementary DNA encoding a new mouse B lymphocyte differentiation antigen, homologous to the human B1 (CD20) antigen, and localization of the gene to chromosome 19. *J Immunol* 141, 4388-94 (1988)

27. Henry C., M. Deschamps, P. S. Rohrlich, J. R. Pallandre, J. P. Remy-Martin, M. Callanan, A. Traverse-Glehen, C. GrandClement, F. Garnache-Ottou, R. Gressin, E. Deconinck, G. Salles, E. Robinet, P. Tiberghien, C. Borg and C. Ferrand: Identification of an alternative CD20 transcript variant in B-cell malignancies coding for a novel protein associated to rituximab resistance. *Blood* 115, 2420-9 (2010)

28. Rieckmann P., G. L. Wilson, C. Thevenin, J. X. Hong and J. H. Kehrl: Analysis of cis-acting elements present in the CD20/B1 antigen promoter. *J Immunol* 147, 3994-9 (1991)

29. Thevenin C., P. Rieckmann, E. J. Kozlow and J. H. Kehrl: Identification of a diverged octamer binding site important in the B cell-specific expression of the CD20 gene. *Trans Assoc Am Physicians* 105, 15-24 (1992)

30. Thevenin C., B. P. Lucas, E. J. Kozlow and J. H. Kehrl: Cell type- and stage-specific expression of the CD20/B1 antigen correlates with the activity of a diverged octamer DNA motif present in its promoter. *J Biol Chem* 268, 5949-56 (1993)

31. Himmelmann A., A. Riva, G. L. Wilson, B. P. Lucas, C. Thevenin and J. H. Kehrl: PU.1/Pip and basic helix loop helix zipper transcription factors interact with binding sites in the CD20 promoter to help confer lineage- and stage-specific expression of CD20 in B lymphocytes. *Blood* 90, 3984-95 (1997)

32. Nutt S. L., D. Eberhard, M. Horcher, A. G. Rolink and M. Busslinger: Pax5 determines the identity of B cells from the beginning to the end of B-lymphopoiesis. *Int Rev Immunol* 20, 65-82 (2001)

33. Dancescu M., C. Wu, M. Rubio, G. Delespesse and M. Sarfati: IL-4 induces conformational change of CD20 antigen via a protein kinase C-independent pathway. Antagonistic effect of anti-CD40 monoclonal antibody. *J Immunol* 148, 2411-6 (1992)

34. Venugopal P., S. Sivaraman, X. K. Huang, J. Nayini, S. A. Gregory and H. D. Preisler: Effects of cytokines on CD20 antigen expression on tumor cells from patients with chronic lymphocytic leukemia. *Leuk Re*, 24, 411-5 (2000)

35. Sivaraman S., C. G. Deshpande, R. Ranganathan, X. Huang, A. Jajeh, T. O'Brien, R. W. Huang, S. A. Gregory, P. Venugopal and H. D. Preisler: Tumor necrosis factor modulates CD 20 expression on cells from chronic lymphocytic leukemia: a new role for TNF alpha? *Microsc Res Tech* 50, 251-7 (2000)

36. Sivaraman S., P. Venugopal, R. Ranganathan, C. G. Deshpande, X. Huang, A. Jajeh, S. A. Gregory, T. O'Brien

and H. D. Preisler: Effect of interferon-alpha on CD20 antigen expression of B-cell chronic lymphocytic leukemia. *Cytokines Cell Mol Ther* 6, 81-7 (2000)

37. Gupta D., M. E. Crosby, A. Almasan and R. M. Macklis: Regulation of CD20 expression by radiation-induced changes in intracellular redox status. *Free Radic Biol Med* 44, 614-23 (2008)

38. Einfeld D. A., J. P. Brown, M. A. Valentine, E. A. Clark and J. A. Ledbetter: Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. *EMBO J*, 7, 711-7 (1988)

39. Ernst J. A., H. Li, H. S. Kim, G. R. Nakamura, D. G. Yansura and R. L. Vandlen: Isolation and characterization of the B-cell marker CD20. *Biochemistry* 44, 15150-8 (2005)

40. Deans J. P., S. M. Robbins, M. J. Polyak and J. A. Savage: Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. J Biol Chem 273, 344-8 (1998)

41. Li H., L. M. Ayer, M. J. Polyak, C. M. Mutch, R. J. Petrie, L. Gauthier, N. Shariat, M. J. Hendzel, A. R. Shaw, K. D. Patel and J. P. Deans: The CD20 calcium channel is localized to microvilli and constitutively associated with membrane rafts: antibody binding increases the affinity of the association through an epitope-dependent cross-linking-independent mechanism. *J Biol Chem* 279, 19893-901 (2004)

42. Janas E., R. Priest, J. I. Wilde, J. H. White and R. Malhotra: Rituxan (anti-CD20 antibody)-induced translocation of CD20 into lipid rafts is crucial for calcium influx and apoptosis. *Clin Exp Immunol* 139, 439-46 (2005)

43. Petrie R. J. and J. P. Deans: Colocalization of the B cell receptor and CD20 followed by activation-dependent dissociation in distinct lipid rafts. *J Immunol* 169, 2886-91 (2002)

44. Leveille C., A. L.-D. R and W. Mourad: CD20 is physically and functionally coupled to MHC class II and CD40 on human B cell lines. *Eur J Immunol* 29, 65-74 (1999)

45. Deans J. P., L. Kalt, J. A. Ledbetter, G. L. Schieven, J. B. Bolen and P. Johnson: Association of 75/80-kDa phosphoproteins and the tyrosine kinases Lyn, Fyn, and Lck with the B cell molecule CD20. Evidence against involvement of the cytoplasmic regions of CD20. *J Biol Chem* 270, 22632-8 (1995)

46. Szöllősi J., V. Hořejší, L. Bene, P. Angelisová and S. Damjanovich: Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. *J Immunol* 157, 2939-46 (1996)

47. Kanzaki M., M. A. Lindorfer, J. C. Garrison and I. Kojima: Activation of the calcium-permeable cation channel CD20 by alpha subunits of the Gi protein. *J Biol Chem* 272, 14733-9 (1997)

48. Holder M., G. Grafton, I. MacDonald, M. Finney and J. Gordon: Engagement of CD20 suppresses apoptosis in germinal center B cells. *Eur J Immunol* 25, 3160-4 (1995)

49. Clark E. A. and G. Shu: Activation of human B cell proliferation through surface Bp35 (CD20) polypeptides or immunoglobulin receptors. *J Immunol* 138, 720-5 (1987)

50. Tedder T. F., A. Forsgren, A. W. Boyd, L. M. Nadler and S. F. Schlossman: Antibodies reactive with the B1 molecule inhibit cell cycle progression but not activation of human B lymphocytes. *Eur J Immunol* 16, 881-7 (1986)

51. Hofmeister J. K., D. Cooney and K. M. Coggeshall: Clustered CD20 induced apoptosis: src-family kinase, the proximal regulator of tyrosine phosphorylation, calcium influx, and caspase 3-dependent apoptosis. *Blood Cells Mol Dis* 26, 133-43 (2000)

52. Cardarelli P. M., M. Quinn, D. Buckman, Y. Fang, D. Colcher, D. J. King, C. Bebbington and G. Yarranton: Binding to CD20 by anti-B1 antibody or F(ab')(2) is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol Immunother* 51, 15-24 (2002)

53. Shan D., J. A. Ledbetter and O. W. Press: Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. *Cancer Immunol Immunother* 48, 673-83 (2000)

54. Walshe C. A., S. A. Beers, R. R. French, C. H. Chan, P. W. Johnson, G. K. Packham, M. J. Glennie and M. S. Cragg: Induction of cytosolic calcium flux by CD20 is dependent upon B Cell antigen receptor signaling. *J Biol Chem* 283, 16971-84 (2008)

55. Li H., L. M. Ayer, J. Lytton and J. P. Deans: Store-operated cation entry mediated by CD20 in membrane rafts. *J Biol Chem* 278, 42427-34 (2003)

56. Uchida J., Y. Lee, M. Hasegawa, Y. Liang, A. Bradney, J. A. Oliver, K. Bowen, D. A. Steeber, K. M. Haas, J. C. Poe and T. F. Tedder: Mouse CD20 expression and function. *Int Immunol* 16, 119-29 (2004)

57. Kuijpers T. W., R. J. Bende, P. A. Baars, A. Grummels, I. A. Derks, K. M. Dolman, T. Beaumont, T. F. Tedder, C. J. van Noesel, E. Eldering and R. A. van Lier: CD20 deficiency in humans results in impaired T cell-independent antibody responses. *J Clin Invest* 120, 214-22

58. Binder M., F. Otto, R. Mertelsmann, H. Veelken and M. Trepel: The epitope recognized by rituximab. *Blood* 108, 1975-8 (2006)

59. Du J., H. Wang, C. Zhong, B. Peng, M. Zhang, B. Li, S. Huo, Y. Guo and J. Ding: Structural basis for recognition

of CD20 by therapeutic antibody Rituximab. *J Biol Chem* 282, 15073-80 (2007)

60. Teeling J. L., W. J. Mackus, L. J. Wiegman, J. H. van den Brakel, S. A. Beers, R. R. French, T. van Meerten, S. Ebeling, T. Vink, J. W. Slootstra, P. W. Parren, M. J. Glennie and J. G. van de Winkel: The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. *J Immunol* 177, 362-71 (2006)

61. Beers S. A., C. H. Chan, S. James, R. R. French, K. E. Attfield, C. M. Brennan, A. Ahuja, M. J. Shlomchik, M. S. Cragg and M. J. Glennie: Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. *Blood* 112, 4170-7 (2008)

62. Cragg M. S. and M. J. Glennie: Antibody specificity controls *in vivo* effector mechanisms of anti-CD20 reagents. *Blood* 103, 2738-43 (2004)

63. Cragg M. S., S. M. Morgan, H. T. Chan, B. P. Morgan, A. V. Filatov, P. W. Johnson, R. R. French and M. J. Glennie: Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood* 101, 1045-52 (2003)

64. Glennie M. J., R. R. French, M. S. Cragg and R. P. Taylor: Mechanisms of killing by anti-CD20 monoclonal antibodies. *Mol Immunol* 44, 3823-37 (2007)

65. Cragg M. S., C. A. Walshe, A. O. Ivanov and M. J. Glennie: The biology of CD20 and its potential as a target for mAb therapy. *Curr Dir Autoimmun* 8, 140-74 (2005)

66. Semac I., C. Palomba, K. Kulangara, N. Klages, G. van Echten-Deckert, B. Borisch and D. C. Hoessli: Anti-CD20 therapeutic antibody rituximab modifies the functional organization of rafts/microdomains of B lymphoma cells. *Cancer Res* 63, 534-40 (2003)

67. Cohen A. M. and M. Shinitzky: Modulation of complement lysis of human erythrocytes by the membrane lipid viscosity. *Vox Sang* 43, 23-7 (1982)

68. Cartron G., H. Watier, J. Golay and P. Solal-Celigny: From the bench to the bedside: ways to improve rituximab efficacy. *Blood* 104, 2635-42 (2004)

69. Idusogie E. E., L. G. Presta, H. Gazzano-Santoro, K. Totpal, P. Y. Wong, M. Ultsch, Y. G. Meng and M. G. Mulkerrin: Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 164, 4178-84 (2000)

70. Polyak M. J., S. H. Tailor and J. P. Deans: Identification of a cytoplasmic region of CD20 required for its redistribution to a detergent-insoluble membrane compartment. *J Immunol* 161, 3242-8 (1998)

71. Dunkelberger J. R. and W. C. Song: Complement and its role in innate and adaptive immune responses. *Cell Res* 20, 34-50 (2010)

72. Ehlenberger A. G. and V. Nussenzweig: The role of membrane receptors for C3b and C3d in phagocytosis. *J Exp Med* 145, 357-71 (1977)

73. Sacks S. H.: Complement fragments C3a and C5a: the salt and pepper of the immune response. *Eur J Immunol* 40, 668-70 (2010)

74. Shushakova N., J. Skokowa, J. Schulman, U. Baumann, J. Zwirner, R. E. Schmidt and J. E. Gessner: C5a anaphylatoxin is a major regulator of activating versus inhibitory FcgammaRs in immune complex-induced lung disease. *J Clin Invest* 110, 1823-30 (2002)

75. Flieger D., S. Renoth, I. Beier, T. Sauerbruch and I. Schmidt-Wolf: Mechanism of cytotoxicity induced by chimeric mouse human monoclonal antibody IDEC-C2B8 in CD20-expressing lymphoma cell lines. *Cell Immunol* 204, 55-63 (2000)

76. Harjunpaa A., S. Junnikkala and S. Meri: Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. *Scand J Immunol* 51, 634-41 (2000)

77. Bellosillo B., N. Villamor, A. Lopez-Guillermo, S. Marce, J. Esteve, E. Campo, D. Colomer and E. Montserrat: 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* 98, 2771-7 (2001)

78. Golay J., M. Lazzari, V. Facchinetti, S. Bernasconi, G. Borleri, T. Barbui, A. Rambaldi and M. Introna: CD20 levels determine the *in vitro* susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 98, 3383-9 (2001)

79. Manches O., G. Lui, L. Chaperot, R. Gressin, J. P. Molens, M. C. Jacob, J. J. Sotto, D. Leroux, J. C. Bensa and J. Plumas: *In vitro* mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 101, 949-54 (2003)

80. van Meerten T., R. S. van Rijn, S. Hol, A. Hagenbeek and S. B. Ebeling: Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity. *Clin Cancer Res* 12, 4027-35 (2006)

81. Cittera E., M. Leidi, C. Buracchi, F. Pasqualini, S. Sozzani, A. Vecchi, J. D. Waterfield, M. Introna and J. Golay: The CCL3 family of chemokines and innate immunity cooperate *in vivo* in the eradication of an established lymphoma xenograft by rituximab. *J Immunol* 178, 6616-23 (2007)

82. Dalle S., S. Dupire, S. Brunet-Manquat, L. Reslan, A. Plesa and C. Dumontet: *In vivo* model of follicular lymphoma resistant to rituximab. *Clin Cancer Res* 15, 851-7 (2009)

83. Golay J., E. Cittera, N. Di Gaetano, M. Manganini, M. Mosca, M. Nebuloni, N. van Rooijen, L. Vago and M. Introna: The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. *Haematologica* 91, 176-83 (2006)

84. Di Gaetano N., E. Cittera, R. Nota, A. Vecchi, V. Grieco, E. Scanziani, M. Botto, M. Introna and J. Golay: Complement activation determines the therapeutic activity of rituximab *in vivo*. *J Immunol* 171, 1581-7 (2003)

85. Kennedy A. D., M. D. Solga, T. A. Schuman, A. W. Chi, M. A. Lindorfer, W. M. Sutherland, P. L. Foley and R. P. Taylor: An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by rituximab. *Blood* 101, 1071-9 (2003)

86. Anderson D. R., A. Grillo-Lopez, C. Varns, K. S. Chambers and N. Hanna: Targeted anti-cancer therapy using rituximab, a chimaeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma. *Biochem Soc Trans* 25, 705-8 (1997)

87. Kennedy A. D., P. V. Beum, M. D. Solga, D. J. DiLillo, M. A. Lindorfer, C. E. Hess, J. J. Densmore, M. E. Williams and R. P. Taylor: Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol* 172, 3280-8 (2004)

88. Winkler U., M. Jensen, O. Manzke, H. Schulz, V. Diehl and A. Engert: Cytokine-release syndrome in patients with B-cell chronic lymphocytic leukemia and high lymphocyte counts after treatment with an anti-CD20 monoclonal antibody (rituximab, IDEC-C2B8). *Blood* 94, 2217-24 (1999)

89. van der Kolk L. E., A. J. Grillo-Lopez, J. W. Baars, C. E. Hack and M. H. van Oers: Complement activation plays a key role in the side-effects of rituximab treatment. *Br J Haematol* 115, 807-11 (2001)

90. Bannerji R., S. Kitada, I. W. Flinn, M. Pearson, D. Young, J. C. Reed and J. C. Byrd: Apoptotic-regulatory and complement-protecting protein expression in chronic lymphocytic leukemia: relationship to *in vivo* rituximab resistance. *J Clin Oncol* 21, 1466-71 (2003)

91. Uchida J., Y. Hamaguchi, J. A. Oliver, J. V. Ravetch, J. C. Poe, K. M. Haas and T. F. Tedder: The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 199, 1659-69 (2004)

92. Beers S. A., R. R. French, C. H. Chan, S. H. Lim, T. C. Jarrett, R. Mora Vidal, S. S. Wijayaweera, S. V. Dixon, H. J. Kim, K. L. Cox, J. P. Kerr, D. A. Johnston, P. W. Johnson, S. Verbeek, M. J. Glennie and M. S. Cragg: Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood* (2010)

93. Minard-Colin V., Y. Xiu, J. C. Poe, M. Horikawa, C. M. Magro, Y. Hamaguchi, K. M. Haas and T. F. Tedder: Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage FcgammaRI, FcgammaRIII, and FcgammaRIV. *Blood* 112, 1205-13 (2008)

94. E. Racila, B. K. Link, W. K. Weng, T. E. Witzig, S. Ansell, M. J. Maurer, J. Huang, C. Dahle, A. Halwani, R. Levy and G. J. Weiner: A polymorphism in the complement component C1qA correlates with prolonged response following rituximab therapy of follicular lymphoma. *Clin Cancer Res* 14, 6697-703 (2008)

95. Klepfish A., L. Gilles, K. Ioannis, R. Eliezer and S. Ami: Enhancing the action of rituximab in chronic lymphocytic leukemia by adding fresh frozen plasma: complement/rituximab interactions & clinical results in refractory CLL. *Ann N Y Acad Sci* 1173, 865-73 (2009)

96. Mishima Y., N. Sugimura, Y. Matsumoto-Mishima, Y. Terui, K. Takeuchi, S. Asai, D. Ennishi, H. Asai, M. Yokoyama, K. Kojima and K. Hatake: An imaging-based rapid evaluation method for complement-dependent cytotoxicity discriminated clinical response to rituximab-containing chemotherapy. *Clin Cancer Res* 15, 3624-32 (2009)

97. Wang S. Y., S. Veeramani, E. Racila, J. Cagley, D. C. Fritzinger, C. W. Vogel, W. St John and G. J. Weiner: 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. *Blood* 114, 5322-30 (2009)

98. Wang S. Y., E. Racila, R. P. Taylor and G. J. Weiner: NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. *Blood* 111, 1456-63 (2008)

99. Verbovetski I., H. Bychkov, U. Trahtemberg, I. Shapira, M. Hareuveni, O. Ben-Tal, I. Kutikov, O. Gill and D. Mevorach: Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. *J Exp Med* 196, 1553-61 (2002)

100. Nimmerjahn F. and J. V. Ravetch: Fcgamma receptors: old friends and new family members. *Immunity* 24, 19-28 (2006)

101. Lim S. H., S. A. Beers, R. R. French, P. W. Johnson, M. J. Glennie and M. S. Cragg: Anti-CD20 monoclonal antibodies: historical and future perspectives. *Haematologica* 95, 135-43 (2010)

102. Schmidt R. E. and J. E. Gessner: Fc receptors and their interaction with complement in autoimmunity. *Immunol Lett* 100, 56-67 (2005)

103. Binyamin L., R. K. Alpaugh, T. L. Hughes, C. T. Lutz, K. S. Campbell and L. M. Weiner: Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. *J Immunol* 180, 6392-401 (2008)

104. Iannello A. and A. Ahmad: Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies. *Cancer Metastasis Rev* 24, 487-99 (2005)

105. Reff M. E., K. Carner, K. S. Chambers, P. C. Chinn, J. E. Leonard, R. Raab, R. A. Newman, N. Hanna and D. R. Anderson: Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83, 435-45 (1994)

106. Bowles J. A., S. Y. Wang, B. K. Link, B. Allan, G. Beuerlein, M. A. Campbell, D. Marquis, B. Ondek, J. E. Wooldridge, B. J. Smith, J. B. Breitmeyer and G. J. Weiner: Anti-CD20 monoclonal antibody with enhanced affinity for CD16 activates NK cells at lower concentrations and more effectively than rituximab. *Blood* 108, 2648-54 (2006)

107. Bhat R. and C. Watzl: Serial killing of tumor cells by human natural killer cells--enhancement by therapeutic antibodies. *PLoS One* 2, e326 (2007)

108. Lefebvre M. L., S. W. Krause, M. Salcedo and A. Nardin: *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* 29, 388-97 (2006)

109. Shimadoi S., A. Takami, Y. Kondo, H. Okumura and S. Nakao: Macrophage colony-stimulating factor enhances rituximab-dependent cellular cytotoxicity by monocytes. *Cancer Sci* 98, 1368-72 (2007)

110. Leidi M., E. Gotti, L. Bologna, E. Miranda, M. Rimoldi, A. Sica, M. Roncalli, G. A. Palumbo, M. Introna and J. Golay: M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells *in vitro*. *J Immunol* 182, 4415-22 (2009)

111. van der Kolk L. E., M. de Haas, A. J. Grillo-Lopez, J. W. Baars and M. H. van Oers: Analysis of CD20-dependent cellular cytotoxicity by G-CSF-stimulated neutrophils. *Leukemia* 16, 693-9 (2002)

112. Tokuyama H., T. Hagi, S. R. Mattarollo, J. Morley, Q. Wang, H. Fai-So, F. Moriyasu, M. Nieda and A. J. Nicol: V gamma 9 V delta 2 T cell cytotoxicity against tumor cells is enhanced by monoclonal antibody drugs--rituximab and trastuzumab. *Int J Cancer* 122, 2526-34 (2008)

113. Inagaki A., T. Ishida, H. Yano, T. Ishii, S. Kusumoto, A. Ito, M. Ri, F. Mori, J. Ding, H. Komatsu, S. Iida and R. Ueda: Expression of the ULBP ligands for NKG2D by B-NHL cells plays an important role in determining their susceptibility to rituximab-induced ADCC. *Int J Cancer* 125, 212-21 (2009)

114. Borgerding A., J. Hasenkamp, M. Engelke, N. Burkhart, L. Trumper, J. Wienands and B. Glass: B-lymphoma cells escape rituximab-triggered elimination by NK cells through increased HLA class I expression. *Exp Hematol* 38, 213-21 (2010)

115. Clynes R. A., T. L. Towers, L. G. Presta and J. V. Ravetch: Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. *Nat Med* 6, 443-6 (2000)

116. Cartron G., L. Dacheux, G. Salles, P. Solal-Celigny, P. Bardos, P. Colombat and H. Watier: Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* 99, 754-8 (2002)

117. Weng W. K. and R. Levy: Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 21, 3940-7 (2003)

118. Treon S. P., M. Hansen, A. R. Branagan, S. Verselis, C. Emmanouilides, E. Kimby, S. R. Frankel, N. Touroutoglou, B. Turnbull, K. C. Anderson, D. G. Maloney and E. A. Fox: Polymorphisms in FcgammaRIIIA (CD16) receptor expression are associated with clinical response to rituximab in Waldenstrom's macroglobulinemia. *J Clin Oncol* 23, 474-81 (2005)

119. Kim D. H., H. D. Jung, J. G. Kim, J. J. Lee, D. H. Yang, Y. H. Park, Y. R. Do, H. J. Shin, M. K. Kim, M. S. Hyun and S. K. Sohn: FCGR3A gene polymorphisms may correlate with response to frontline R-CHOP therapy for diffuse large B-cell lymphoma. *Blood* 108, 2720-5 (2006)

120. Carlotti E., G. A. Palumbo, E. Oldani, D. Tibullo, S. Salmoiraghi, A. Rossi, J. Golay, A. Pulsoni, R. Foa and A. Rambaldi: FcgammaRIIIA and FcgammaRIIA polymorphisms do not predict clinical outcome of follicular non-Hodgkin's lymphoma patients treated with sequential CHOP and rituximab. *Haematologica* 92, 1127-30 (2007)

121. Rossi D., S. Rasi, S. Franceschetti, D. Capello, A. Castelli, L. De Paoli, A. Ramponi, A. Chiappella, E. M. Pogliani, U. Vitolo, I. Kwee, F. Bertoni, A. Conconi and G. Gaidano: Analysis of the host pharmacogenetic background for prediction of outcome and toxicity in diffuse large B-cell lymphoma treated with R-CHOP21. *Leukemia* 23, 1118-26 (2009)

122. Farag S. S., I. W. Flinn, R. Modali, T. A. Lehman, D. Young and J. C. Byrd: Fc gamma RIIIa and Fc gamma RIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. *Blood* 103, 1472-4 (2004)

123. Hale G., M. Clark and H. Waldmann: Therapeutic potential of rat monoclonal antibodies: isotype specificity

of antibody-dependent cell-mediated cytotoxicity with human lymphocytes. *J Immunol* 134, 3056-61 (1985)

124. Weng W. K. and R. Levy: Genetic polymorphism of the inhibitory IgG Fc receptor FcgammaRIIb is not associated with clinical outcome in patients with follicular lymphoma treated with rituximab. *Leuk Lymphoma* 50, 723-7 (2009)

125. Hamaguchi Y., Y. Xiu, K. Komura, F. Nimmerjahn and T. F. Tedder: Antibody isotype-specific engagement of Fcgamma receptors regulates B lymphocyte depletion during CD20 immunotherapy. *J Exp Med* 203, 743-53 (2006)

126. Taskinen M., M. L. Karjalainen-Lindsberg, H. Nyman, L. M. Eerola and S. Leppa: A high tumor-associated macrophage content predicts favorable outcome in follicular lymphoma patients treated with rituximab and cyclophosphamide-doxorubicin-vincristine-prednisone. *Clin Cancer Res* 13, 5784-9 (2007)

127. Canioni D., G. Salles, N. Mounier, N. Brousse, M. Keuppens, F. Morchhauser, T. Lamy, A. Sonet, M. C. Rousselet, C. Foussard and L. Xerri: High numbers of tumor-associated macrophages have an adverse prognostic value that can be circumvented by rituximab in patients with follicular lymphoma enrolled onto the GELA-GOELAMS FL-2000 trial. *J Clin Oncol* 26, 440-6 (2008)

128. Elsasser D., T. Valerius, R. Repp, G. J. Weiner, Y. Deo, J. R. Kalden, J. G. van de Winkel, G. T. Stevenson, M. J. Glennie and M. Gramatzki: HLA class II as potential target antigen on malignant B cells for therapy with bispecific antibodies in combination with granulocyte colony-stimulating factor. *Blood* 87, 3803-12 (1996)

129. Hernandez-Ilizaliturri F. J., V. Jupudy, J. Ostberg, E. Oflazoglu, A. Huberman, E. Repasky and M. S. Czuczman: Neutrophils contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. *Clin Cancer Res* 9, 5866-73 (2003)

130. Cartron G., M. Ohresser, G. Salles, P. Solal-Celigny, P. Colombat and H. Watier: Neutrophil role in *in vivo* anti-lymphoma activity of rituximab: FCGR3B-NA1/NA2 polymorphism does not influence response and survival after rituximab treatment. *Ann Oncol* 19, 1485-7 (2008)

131. Hotchkiss R. S., A. Strasser, J. E. McDunn and P. E. Swanson: Cell death. *N Engl J Med* 361, 1570-83 (2009)

132. Garg A. D., D. Nowis, J. Golab, P. Vandenabeele, D. V. Krysko and P. Agostinis: Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. *Biochim Biophys Acta* 1805, 53-71 (2010)

133. Cho Y. S., S. Challa, D. Moquin, R. Genga, T. D. Ray, M. Guildford and F. K. Chan: Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137, 1112-23 (2009)

134. Pedersen I. M., A. M. Buhl, P. Klausen, C. H. Geisler and J. Jurlander: 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* 99, 1314-9 (2002)

135. Shan D., J. A. Ledbetter and O. W. Press: Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood* 91, 1644-52 (1998)

136. Ghetie M. A., H. Bright and E. S. Vitetta: Homodimers but not monomers of Rituxan (chimeric anti-CD20) induce apoptosis in human B-lymphoma cells and synergize with a chemotherapeutic agent and an immunotoxin. *Blood* 97, 1392-8 (2001)

137. Bingaman M. G., G. D. Basu, T. C. Golding, S. K. Chong, A. J. Lassen, T. J. Kindt and C. A. Lipinski: The autophilic anti-CD20 antibody DXL625 displays enhanced potency due to lipid raft-dependent induction of apoptosis. *Anticancer Drugs* 21, 532-42 (2010)

138. Zhang N., L. A. Khawli, P. Hu and A. L. Epstein: Generation of rituximab polymer may cause hyper-cross-linking-induced apoptosis in non-Hodgkin's lymphomas. *Clin Cancer Re*, 11, 5971-80 (2005)

139. McLaughlin P., A. J. Grillo-Lopez, B. K. Link, R. Levy, M. S. Czuczman, M. E. Williams, M. R. Heyman, I. Bence-Bruckler, C. A. White, F. Cabanillas, V. Jain, A. D. Ho, J. Lister, K. Wey, D. Shen and B. K. Dallaire: Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 16, 2825-33 (1998)

140. de Haij S., J. H. Jansen, P. Boross, F. J. Beurskens, J. E. Bakema, D. L. Bos, A. Martens, J. S. Verbeek, P. W. Parren, J. G. van de Winkel and J. H. Leusen: *In vivo* cytotoxicity of type I CD20 antibodies critically depends on Fc receptor ITAM signaling. *Cancer Res* 70, 3209-17 (2010)

141. Byrd J. C., S. Kitada, I. W. Flinn, J. L. Aron, M. Pearson, D. Lucas and J. C. Reed: The mechanism of tumor cell clearance by rituximab *in vivo* in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood* 99, 1038-43 (2002)

142. Stolz C., G. Hess, P. S. Hahnel, F. Grabellus, S. Hoffarth, K. W. Schmid and M. Schuler: Targeting Bcl-2 family proteins modulates the sensitivity of B-cell lymphoma to rituximab-induced apoptosis. *Blood* 112, 3312-21 (2008)

143. Stel A. J., B. Ten Cate, S. Jacobs, J. W. Kok, D. C. Spierings, M. Dondorff, W. Helfrich, H. C. Kluin-Nelemans, L. F. de Leij, S. Withoff and B. J. Kroesen: Fas receptor clustering and involvement of the death receptor pathway in rituximab-mediated apoptosis with concomitant sensitization of lymphoma B cells to fas-induced apoptosis. *J Immunol* 178, 2287-95 (2007)

144. Eeva J., U. Nuutinen, A. Ropponen, M. Matto, M. Eray, R. Pellinen, J. Wahlfors and J. Pelkonen: The involvement of mitochondria and the caspase-9 activation pathway in rituximab-induced apoptosis in FL cells. *Apoptosis* 14, 687-98 (2009)

145. Stanglmaier M., S. Reis and M. Hallek: Rituximab and alemtuzumab induce a nonclassic, caspase-independent apoptotic pathway in B-lymphoid cell lines and in chronic lymphocytic leukemia cells. *Ann Hematol* 83, 634-45 (2004)

146. van der Kolk L. E., L. M. Evers, C. Omene, S. M. Lens, S. Lederman, R. A. van Lier, M. H. van Oers and E. Eldering: CD20-induced B cell death can bypass mitochondria and caspase activation. *Leukemia* 16, 1735-44 (2002)

147. Vega M. I., S. Huerta-Yepez, A. R. Jazirehi, H. Garban and B. Bonavida: Rituximab (chimeric anti-CD20) sensitizes B-NHL cell lines to Fas-induced apoptosis. *Oncogene* 24, 8114-27 (2005)

148. Maddipatla S., F. J. Hernandez-Ilizaliturri, J. Knight and M. S. Czuczman: Augmented antitumor activity against B-cell lymphoma by a combination of monoclonal antibodies targeting TRAIL-R1 and CD20. *Clin Cancer Res* 13, 4556-64 (2007)

149. Daniel D., B. Yang, D. A. Lawrence, K. Totpal, I. Balter, W. P. Lee, A. Gogineni, M. J. Cole, S. F. Yee, S. Ross and A. Ashkenazi: Cooperation of the proapoptotic receptor agonist rhApo2L/TRAIL with the CD20 antibody rituximab against non-Hodgkin lymphoma xenografts. *Blood* 110, 4037-46 (2007)

150. Bezombes C., S. Grazide, C. Garret, C. Fabre, A. Quillet-Mary, S. Muller, J. P. Jaffrezou and G. Laurent: Rituximab antiproliferative effect in B-lymphoma cells is associated with acid-sphingomyelinase activation in raft microdomains. *Blood* 104, 1166-73 (2004)

151. Daniels I., A. M. Abulayha, B. J. Thomson and A. P. Haynes: Caspase-independent killing of Burkitt lymphoma cell lines by rituximab. *Apoptosis* 11, 1013-23 (2006)

152. Meyer zum Buschenfelde C., Y. Feuerstacke, K. S. Gotze, K. Scholze and C. Peschel: GM1 expression of non-Hodgkin's lymphoma determines susceptibility to rituximab treatment. *Cancer Res* 68, 5414-22 (2008)

153. Chan H. T., D. Hughes, R. R. French, A. L. Tutt, C. A. Walshe, J. L. Teeling, M. J. Glennie and M. S. Cragg: CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. *Cancer Res* 63, 5480-9 (2003)

154. Ivanov A., S. A. Beers, C. A. Walshe, J. Honeychurch, W. Alduaij, K. L. Cox, K. N. Potter, S. Murray, C. H. Chan, T. Klymenko, J. Erenpreisa, M. J. Glennie, T. M. Illidge and M. S. Cragg: Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. *J Clin Invest* 119, 2143-59 (2009)

155. Smith M. R., F. Jin and I. Joshi: Enhanced efficacy of therapy with antisense BCL-2 oligonucleotides plus anti-CD20 monoclonal antibody in scid mouse/human lymphoma xenografts. *Mol Cancer Ther* 3, 1693-9 (2004)

156. Paoluzzi L., M. Gonen, J. R. Gardner, J. Mastrella, D. Yang, J. Holmlund, M. Sorensen, L. Leopold, K. Manova, G. Marcucci, M. L. Heaney and O. A. O'Connor: Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in *in vitro* and *in vivo* models of B-cell lymphoma. *Blood* 111, 5350-8 (2008)

157. Pro B., B. Leber, M. Smith, L. Fayad, J. Romaguera, F. Hagemeister, A. Rodriguez, P. McLaughlin, F. Samaniego, J. Zwiebel, A. Lopez, L. Kwak and A. Younes: Phase II multicenter study of oblimersen sodium, a Bcl-2 antisense oligonucleotide, in combination with rituximab in patients with recurrent B-cell non-Hodgkin lymphoma. *Br J Haematol* 143, 355-60 (2008)

158. Rafiq K., A. Bergtold and R. Clynes: Immune complex-mediated antigen presentation induces tumor immunity. *J Clin Invest* 110, 71-9 (2002)

159. Peng Q., K. Li, N. Wang, Q. Li, E. Asgari, B. Lu, T. M. Woodruff, S. H. Sacks and W. Zhou: Dendritic cell function in allostimulation is modulated by C5aR signaling. *J Immunol* 183, 6058-68 (2009)

160. Li K., K. J. Anderson, Q. Peng, A. Noble, B. Lu, A. P. Kelly, N. Wang, S. H. Sacks and W. Zhou: Cyclic AMP plays a critical role in C3a-receptor-mediated regulation of dendritic cells in antigen uptake and T-cell stimulation. *Blood* 112, 5084-94 (2008)

161. Selenko N., O. Maidic, S. Draxier, A. Berer, U. Jager, W. Knapp and J. Stockl: CD20 antibody (C2B8)-induced apoptosis of lymphoma cells promotes phagocytosis by dendritic cells and cross-priming of CD8+ cytotoxic T cells. *Leukemia* 15, 1619-26 (2001)

162. Gadri Z., T. Kukulansky, E. Bar-Or, J. Haimovich and N. Hollander: Synergistic effect of dendritic cell vaccination and anti-CD20 antibody treatment in the therapy of murine lymphoma. *J Immunother* 32, 333-40 (2009)

163. Hilchey S. P., O. Hyrien, T. R. Mosmann, A. M. Livingstone, J. W. Friedberg, F. Young, R. I. Fisher, R. J.

Kelleher, Jr., R. B. Bankert and S. H. Bernstein: Rituximab immunotherapy results in the induction of a lymphoma idiotype-specific T-cell response in patients with follicular lymphoma: support for a "vaccinal effect" of rituximab. *Blood* 113, 3809-12 (2009)

164. Davis T. A., A. J. Grillo-Lopez, C. A. White, P. McLaughlin, M. S. Czuczman, B. K. Link, D. G. Maloney, R. L. Weaver, J. Rosenberg and R. Levy: Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J Clin Oncol* 18, 3135-43 (2000)

165. Hainsworth J. D., S. Litchy, D. W. Shaffer, V. L. Lackey, M. Grimaldi and F. A. Greco: Maximizing therapeutic benefit of rituximab: maintenance therapy versus re-treatment at progression in patients with indolent non-Hodgkin's lymphoma--a randomized phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol* 23, 1088-95 (2005)

166. Ollert M. W., J. V. Kadlec, K. David, E. C. Petrella, R. Bredehorst and C. W. Vogel: Antibody-mediated complement activation on nucleated cells. A quantitative analysis of the individual reaction steps. *J Immunol* 153, 2213-21 (1994)

167. Koski C. L., L. E. Ramm, C. H. Hammer, M. M. Mayer and M. L. Shin: Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics. *Proc Natl Acad Sci U S A* 80, 3816-20 (1983)

168. Seliem R. M., J. K. Freeman, R. H. Steingart and R. P. Hasserjian: Immunophenotypic changes and clinical outcome in B-cell lymphomas treated with rituximab. *Appl Immunohistochem Mol Morphol* 14, 18-23 (2006)

169. Davis T. A., D. K. Czerwinski and R. Levy: Therapy of B-cell lymphoma with anti-CD20 antibodies can result in the loss of CD20 antigen expression. *Clin Cancer Res* 5, 611-5 (1999)

170. Foran J. M., A. J. Norton, I. N. Micallef, D. C. Taussig, J. A. Amess, A. Z. Rohatiner and T. A. Lister: Loss of CD20 expression following treatment with rituximab (chimaeric monoclonal anti-CD20): a retrospective cohort analysis. *Br J Haematol* 114, 881-3 (2001)

171. Schmitz K., W. Brugger, B. Weiss, E. Kaiserling and L. Kanz: Clonal selection of CD20-negative non-Hodgkin's lymphoma cells after treatment with anti-CD20 antibody rituximab. *Br J Haematol* 106, 571-2 (1999)

172. Kinoshita T., H. Nagai, T. Murate and H. Saito: CD20-negative relapse in B-cell lymphoma after treatment with Rituximab. *J Clin Oncol* 16, 3916 (1998)

173. Haidar J. H., A. Shamseddine, Z. Salem, Y. A. Mrad, M. R. Nasr, G. Zaatari and A. Bazarbachi: Loss of CD20 expression in relapsed lymphomas after rituximab therapy. *Eur J Haematol* 70, 330-2 (2003)

174. Maeshima A. M., H. Taniguchi, J. Nomoto, D. Maruyama, S. W. Kim, T. Watanabe, Y. Kobayashi, K. Tobinai and Y. Matsuno: Histological and immunophenotypic changes in 59 cases of B-cell non-Hodgkin's lymphoma after rituximab therapy. *Cancer Sci* 100, 54-61 (2009)

175. Hiraga J., A. Tomita, T. Sugimoto, K. Shimada, M. Ito, S. Nakamura, H. Kiyoi, T. Kinoshita and T. Naoe: Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. *Blood* 113, 4885-93 (2009)

176. Jilani I., S. O'Brien, T. Manshuri, D. A. Thomas, V. A. Thomazy, M. Imam, S. Naeem, S. Verstovsek, H. Kantarjian, F. Giles, M. Keating and M. Albitar: Transient down-modulation of CD20 by rituximab in patients with chronic lymphocytic leukemia. *Blood* 102, 3514-20 (2003)

177. Pickartz T., F. Ringel, M. Wedde, H. Renz, A. Klein, N. von Neuhoff, P. Dreger, K. A. Kreuzer, C. A. Schmidt, S. Srock, D. Schoeler and F. Schriever: Selection of B-cell chronic lymphocytic leukemia cell variants by therapy with anti-CD20 monoclonal antibody rituximab. *Exp Hematol* 29, 1410-6 (2001)

178. Mankai A., A. Bordron, Y. Renaudineau, C. Martins-Carvalho, S. Takahashi, I. Ghedira, C. Berthou and P. Youinou: Purine-rich box-1-mediated reduced expression of CD20 alters rituximab-induced lysis of chronic lymphocytic leukemia B cells. *Cancer Res* 68, 7512-9 (2008)

179. Ushmorov A., F. Leithauser, O. Sakk, A. Weinhausel, S. W. Popov, P. Moller and T. Wirth: Epigenetic processes play a major role in B-cell-specific gene silencing in classical Hodgkin lymphoma. *Blood*, 107, 2493-500 (2006)

180. T. Sugimoto, A. Tomita, J. Hiraga, K. Shimada, H. Kiyoi, T. Kinoshita and T. Naoe: Escape mechanisms from antibody therapy to lymphoma cells: downregulation of CD20 mRNA by recruitment of the HDAC complex and not by DNA methylation. Biochem Biophys Res Commun 390, 48-53 (2009)

181. Tomita A., J. Hiraga, H. Kiyoi, M. Ninomiya, T. Sugimoto, M. Ito, T. Kinoshita and T. Naoe: Epigenetic regulation of CD20 protein expression in a novel B-cell lymphoma cell line, RRBL1, established from a patient treated repeatedly with rituximab-containing chemotherapy. Int J Hematol 86, 49-57 (2007)

182. Michel R. B. and M. J. Mattes: Intracellular accumulation of the anti-CD20 antibody 1F5 in B-lymphoma cells. *Clin Cancer Res* 8, 2701-13 (2002)

183. Michel R. B., R. Ochakovskaya and M. J. Mattes: Antibody localization to B-cell lymphoma xenografts in immunodeficient mice: importance of using residualizing radiolabels. *Clin Cancer Res* 8, 2632-9 (2002)

184. Turzanski J., I. Daniels and A. P. Haynes: Internalisation of uncross-linked rituximab is not essential for the induction of caspase-independent killing in Burkitt lymphoma cell lines. *Leuk Lymphoma* 49, 1578-91 (2008)

185. Anolik J., R. J. Looney, A. Bottaro, I. Sanz and F. Young: Down-regulation of CD20 on B cells upon CD40 activation. *Eur J Immunol* 33, 2398-409 (2003)

186. Lapalombella R., B. Yu, G. Triantafillou, Q. Liu, J. P. Butchar, G. Lozanski, A. Ramanunni, L. L. Smith, W. Blum, L. Andritsos, D. S. Wang, A. Lehman, C. S. Chen, A. J. Johnson, G. Marcucci, R. J. Lee, L. J. Lee, S. Tridandapani, N. Muthusamy and J. C. Byrd: Lenalidomide down-regulates the CD20 antigen and antagonizes direct and antibody-dependent cellular cytotoxicity of rituximab on primary chronic lymphocytic leukemia cells. *Blood* 112, 5180-9 (2008)

187. Giles F. J., J. M. Vose, K. A. Do, M. M. Johnson, T. Manshouri, G. Bociek, P. J. Bierman, S. M. O'Brien, M. J. Keating, H. M. Kantarjian, J. O. Armitage and M. Albitar: Circulating CD20 and CD52 in patients with non-Hodgkin's lymphoma or Hodgkin's disease. *Br J Haematol* 123, 850-7 (2003)

188. Alatrash G., M. Albitar, S. O'Brien, X. Wang, T. Manshouri, S. Faderl, A. Ferrajoli, J. Burger, G. Garcia-Manero, H. M. Kantarjian, S. Lerner, M. J. Keating and W. G. Wierda: Circulating CD52 and CD20 levels at end of treatment predict for progression and survival in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab (FCR). *Br J Haematol*, 148, 386-93 (2010)

189. Manshouri T., K. A. Do, X. Wang, F. J. Giles, S. M. O'Brien, H. Saffer, D. Thomas, I. Jilani, H. M. Kantarjian, M. J. Keating and M. Albitar: Circulating CD20 is detectable in the plasma of patients with chronic lymphocytic leukemia and is of prognostic significance. *Blood* 101, 2507-13 (2003)

190. Czuczman M. S., S. Olejniczak, A. Gowda, A. Kotowski, A. Binder, H. Kaur, J. Knight, P. Starostik, J. Deans and F. J. Hernandez-Ilizaliturri: Acquirement 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* 14, 1561-70 (2008)

191. Bil J., M. Winiarska, D. Nowis, K. Bojarczuk, A. Dabrowska-Iwanicka, G. W. Basak, K. Sulek, M. Jakobisiak and J. Golab: Bortezomib modulates surface CD20 in B-cell malignancies and affects rituximab-mediated complement-dependent cytotoxicity. *Blood* 115, 3745-3755 (2010)

192. Winiarska M., J. Bil, E. Wilczek, G. M. Wilczynski, M. Lekka, P. J. Engelberts, W. J. Mackus, E. Gorska, L. Bojarski, T. Stoklosa, D. Nowis, Z. Kurzaj, M. Makowski, E. Glodkowska, T. Issat, P. Mrowka, W. Lasek, A. Dabrowska-Iwanicka, G. W. Basak, M. Wasik, K. Warzocha, M. Sinski, Z. Gaciong, M. Jakobisiak, P. W. Parren and J. Golab: Statins impair antitumor effects of rituximab by inducing conformational changes of CD20. *PLoS Med* 5, e64 (2008)

193. Terui Y., Y. Mishima, N. Sugimura, K. Kojima, T. Sakurai, R. Kuniyoshi, A. Taniyama, M. Yokoyama, S. Sakajiri, K. Takeuchi, C. Watanabe, S. Takahashi, Y. Ito and K. Hatake: Identification of CD20 C-terminal deletion mutations associated with loss of CD20 expression in non-Hodgkin's lymphoma. *Clin Cancer Res* 15, 2523-30 (2009)

194. Johnson N. A., S. Leach, B. Woolcock, R. J. deLeeuw, A. Bashashati, L. H. Sehn, J. M. Connors, M. Chhanabhai, A. Brooks-Wilson and R. D. Gascoyne: CD20 mutations involving the rituximab epitope are rare in diffuse large B-cell lymphomas and are not a significant cause of R-CHOP failure. *Haematologica* 94, 423-7 (2009)

195. Sar A., M. Perizzolo, D. Stewart, A. Mansoor, L. M. Difrancesco and D. J. Demetrick: Mutation or polymorphism of the CD20 gene is not associated with the response to R-CHOP in diffuse large B cell lymphoma patients. *Leuk Re*, 33, 792-7 (2009)

196. Bowles J. A. and G. J. Weiner: CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. *J Immunol Methods* 304, 88-99 (2005)

197. Varchetta S., N. Gibelli, B. Oliviero, E. Nardini, R. Gennari, G. Gatti, L. S. Silva, L. Villani, E. Tagliabue, S. Menard, A. Costa and F. F. Fagnoni: Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. *Cancer Res* 67, 11991-9 (2007)

198. Berdeja J. G., A. Hess, D. M. Lucas, P. O'Donnell, R. F. Ambinder, L. F. Diehl, D. Carter-Brookins, S. Newton and I. W. Flinn: Systemic interleukin-2 and adoptive transfer of lymphokine-activated killer cells improves antibody-dependent cellular cytotoxicity in patients with relapsed B-cell lymphoma treated with rituximab. *Clin Cancer Res* 13, 2392-9 (2007)

199. Taylor R. P. and M. A. Lindorfer: Antigenic modulation and rituximab resistance. *Semin Hematol* 47, 124-32 (2010)

200. Beum P. V., A. D. Kennedy, M. E. Williams, M. A. Lindorfer and R. P. Taylor: The shaving reaction: rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes. *J Immunol* 176, 2600-9 (2006)

201. Taylor R. P. and M. A. Lindorfer: Immunotherapeutic mechanisms of anti-CD20 monoclonal antibodies. *Curr Opin Immunol* 20, 444-9 (2008)

202. Williams M. E., J. J. Densmore, A. W. Pawluczkowycz, P. V. Beum, A. D. Kennedy, M. A. Lindorfer, S. H. Hamil, J. C. Eggleton and R. P. Taylor:

Thrice-weekly low-dose rituximab decreases CD20 loss via shaving and promotes enhanced targeting in chronic lymphocytic leukemia. *J Immuno*, 177, 7435-43 (2006)

203. Aue G., M. A. Lindorfer, P. V. Beum, A. W. Pawluczkowycz, B. Vire, T. Hughes, R. P. Taylor and A. Wiestner: Fractionated subcutaneous rituximab is well-tolerated and preserves CD20 expression on tumor cells in patients with chronic lymphocytic leukemia. *Haematologica* 95, 329-32 (2010)

204. Takei K., T. Yamazaki, U. Sawada, H. Ishizuka and S. Aizawa: Analysis of changes in CD20, CD55, and CD59 expression on established rituximab-resistant B-lymphoma cell lines. *Leuk Res* 30, 625-31 (2006)

205. Zhou X., W. Hu and X. Qin: The role of complement in the mechanism of action of rituximab for B-cell lymphoma: implications for therapy. *Oncologist* 13, 954-66 (2008)

206. Bladergroen B. A., C. J. Meijer, R. L. ten Berge, C. E. Hack, J. J. Muris, D. F. Dukers, A. Chott, Y. Kazama, J. J. Oudejans, O. van Berkum and J. A. Kummer: Expression of the granzyme B inhibitor, protease inhibitor 9, by tumor cells in patients with non-Hodgkin and Hodgkin lymphoma: a novel protective mechanism for tumor cells to circumvent the immune system? *Blood* 99, 232-7 (2002)

207. Golay J., L. Zaffaroni, T. Vaccari, M. Lazzari, G. M. Borleri, S. Bernasconi, F. Tedesco, A. Rambaldi and M. Introna: Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 95, 3900-8 (2000)

208. Terui Y., T. Sakurai, Y. Mishima, N. Sugimura, C. Sasaoka, K. Kojima, M. Yokoyama, N. Mizunuma, S. Takahashi, Y. Ito and K. Hatake: Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab. *Cancer Sci* 97, 72-9 (2006)

209. Ziller F., P. Macor, R. Bulla, D. Sblattero, R. Marzari and F. Tedesco: Controlling complement resistance in cancer by using human monoclonal antibodies that neutralize complement-regulatory proteins CD55 and CD59. *Eur J Immunol* 35, 2175-83 (2005)

210. Nagajothi N., W. H. Matsui, G. L. Mukhina and R. A. Brodsky: Enhanced cytotoxicity of rituximab following genetic and biochemical disruption of glycosylphosphatidylinositol anchored proteins. *Leuk Lymphoma* 45, 795-9 (2004)

211. Macor P., C. Tripodo, S. Zorzet, E. Piovan, F. Bossi, R. Marzari, A. Amadori and F. Tedesco: *In vivo* targeting of human neutralizing antibodies against CD55 and CD59 to lymphoma cells increases the antitumor activity of rituximab. *Cancer Res* 67, 10556-63 (2007)

212. Wang H., Y. Liu, Z. Y. Li, X. Fan, A. Hemminki and A. Lieber: A recombinant adenovirus type 35 fiber knob protein sensitizes lymphoma cells to rituximab therapy. *Blood* 115, 592-600 (2010)

213. Di Gaetano N., Y. Xiao, E. Erba, R. Bassan, A. Rambaldi, J. Golay and M. Introna: Synergism between fludarabine and rituximab revealed in a follicular lymphoma cell line resistant to the cytotoxic activity of either drug alone. *Br J Haematol* 114, 800-9 (2001)

214. Paas Y., O. Bohana-Kashtan and Z. Fishelson: Phosphorylation of the complement component, C9, by an ecto-protein kinase of human leukemic cells. *Immunopharmacology* 42, 175-85 (1999)

215. Bohana-Kashtan O., L. A. Pinna and Z. Fishelson: Extracellular phosphorylation of C9 by protein kinase CK2 regulates complement-mediated lysis. *Eur J Immunol* 35, 1939-48 (2005)

216. Peng W., X. Zhang, N. Mohamed, G. Inghirami, K. Takeshita, A. Pecora, L. L. Nardone, S. E. Pincus, L. S. Casey and G. L. Spitalny: A DeImmunized chimeric anti-C3b/iC3b monoclonal antibody enhances rituximab-mediated killing in NHL and CLL cells via complement activation. *Cancer Immunol Immunother* 54, 1172-9 (2005)

217. Weng W. K. and R. Levy: 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* 98, 1352-7 (2001)

218. Gowda A., J. Roda, S. R. Hussain, A. Ramanunni, T. Joshi, S. Schmidt, X. Zhang, A. Lehman, D. Jarjoura, W. E. Carson, W. Kindsvogel, C. Cheney, M. A. Caligiuri, S. Tridandapani, N. Muthusamy and J. C. Byrd: IL-21 mediates apoptosis through up-regulation of the BH3 family member BIM and enhances both direct and antibody-dependent cellular cytotoxicity in primary chronic lymphocytic leukemia cells *in vitro*. *Blood*, 111, 4723-30 (2008)

219. Moga E., E. Alvarez, E. Canto, S. Vidal, J. L. Rodriguez-Sanchez, J. Sierra and J. Briones: NK cells stimulated with IL-15 or CpG ODN enhance rituximab-dependent cellular cytotoxicity against B-cell lymphoma. *Exp Hematol* 36, 69-77 (2008)

220. Voso M. T., G. Pantel, S. Rutella, M. Weis, F. D'Alo, R. Urbano, G. Leone, R. Haas and S. Hohaus: Rituximab reduces the number of peripheral blood B-cells *in vitro* mainly by effector cell-mediated mechanisms. *Haematologica* 87, 918-25 (2002)

221. Niitsu N., M. Hayama, M. Okamoto, M. Khori, M. Higashihara, J. Tamaru and M. Hirano: Phase I study of Rituximab-CHOP regimen in combination with granulocyte colony-stimulating factor in patients with follicular lymphoma. *Clin Cancer Res* 10, 4077-82 (2004)

222. van der Kolk L. E., A. J. Grillo-Lopez, J. W. Baars and M. H. van Oers: Treatment of relapsed B-cell non-Hodgkin's lymphoma with a combination of chimeric anti-CD20 monoclonal antibodies (rituximab) and G-CSF: final report on safety and efficacy. *Leukemia* 17, 1658-64 (2003)

223. Cartron G., L. Zhao-Yang, M. Baudard, T. Kanouni, V. Rouille, P. Quittet, B. Klein and J. F. Rossi: Granulocyte-macrophage colony-stimulating factor potentiates rituximab in patients with relapsed follicular lymphoma: results of a phase II study. *J Clin Oncol* 26, 2725-31 (2008)

224. Khan K. D., C. Emmanouilides, D. M. Benson, Jr., D. Hurst, P. Garcia, G. Michelson, S. Milan, A. K. Ferketich, L. Piro, J. P. Leonard, P. Porcu, C. F. Eisenbeis, A. L. Banks, L. Chen, J. C. Byrd and M. A. Caligiuri: A phase 2 study of rituximab in combination with recombinant interleukin-2 for rituximab-refractory indolent non-Hodgkin's lymphoma. *Clin Cancer Res* 12, 7046-53 (2006)

225. Morito T., M. Fujihara, H. Asaoku, A. Tari, Y. Sato, K. Ichimura, T. Tanaka, K. Takata, M. Tamura and T. Yoshino: Serum soluble interleukin-2 receptor level and immunophenotype are prognostic factors for patients with diffuse large B-cell lymphoma. *Cancer Sci* 100, 1255-60 (2009)

226. Golab J. and R. Zagozdzon: Antitumor effects of interleukin-12 in pre-clinical and early clinical studies (Review). *Int J Mol Med* 3, 537-44 (1999)

227. Ansell S. M., S. M. Geyer, M. J. Maurer, P. J. Kurtin, I. N. Micallef, P. Stella, P. Etzell, A. J. Novak, C. Erlichman and T. E. Witzig: Randomized phase II study of interleukin-12 in combination with rituximab in previously treated non-Hodgkin's lymphoma patients. *Clin Cancer Re*, 12, 6056-63 (2006)

228. Davis T. A., D. G. Maloney, A. J. Grillo-Lopez, C. A. White, M. E. Williams, G. J. Weiner, S. Dowden and R. Levy: Combination immunotherapy of relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma with rituximab and interferon-alpha-2a. *Clin Cancer Re*, 6, 2644-52 (2000)

229. Betting D. J., R. E. Yamada, K. Kafi, J. Said, N. van Rooijen and J. M. Timmerman: Intratumoral but not systemic delivery of CpG oligodeoxynucleotide augments the efficacy of anti-CD20 monoclonal antibody therapy against B cell lymphoma. *J Immunother* 32, 622-31 (2009)

230. Modak S., G. Koehne, A. Vickers, R. J. O'Reilly and N. K. Cheung: Rituximab therapy of lymphoma is enhanced by orally administered (1-->3),(1-->4)-D-beta-glucan. *Leuk Res* 29, 679-83 (2005)

231. Wu L., M. Adams, T. Carter, R. Chen, G. Muller, D. Stirling, P. Schafer and J. B. Bartlett: lenalidomide enhances natural killer cell and monocyte-mediated antibody-dependent cellular cytotoxicity of rituximab-treated CD20+ tumor cells. *Clin Cancer Res* 14, 4650-7 (2008)

232. Romaguera J. E., L. Fayad, M. A. Rodriguez, K. R. Broglio, F. B. Hagemeister, B. Pro, P. McLaughlin, A. Younes, F. Samaniego, A. Goy, A. H. Sarris, N. H. Dang, M. Wang, V. Beasley, L. J. Medeiros, R. L. Katz, H. Gagneja, B. I. Samuels, T. L. Smith and F. F. Cabanillas: High rate of durable remissions after treatment of newly diagnosed aggressive mantle-cell lymphoma with rituximab plus hyper-CVAD alternating with rituximab plus high-dose methotrexate and cytarabine. *J Clin Oncol* 23, 7013-23 (2005)

233. Tam C. S., M. Wolf, H. M. Prince, E. H. Januszewicz, D. Westerman, K. I. Lin, D. Carney and J. F. Seymour: Fludarabine, cyclophosphamide, and rituximab for the treatment of patients with chronic lymphocytic leukemia or indolent non-Hodgkin lymphoma. *Cancer* 106, 2412-20 (2006)

234. Wohrer S., M. Troch, J. Zwerina, G. Schett, C. Skrabs, A. Gaiger, U. Jaeger, C. C. Zielinski and M. Raderer: Influence of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone on serologic parameters and clinical course in lymphoma patients with autoimmune diseases. *Ann Onco*, 18, 647-51 (2007)

235. Jazirehi A. R., X. H. Gan, S. De Vos, C. Emmanouilides and B. Bonavida: 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* 2, 1183-93 (2003)

236. Zwick C., J. Birkmann, N. Peter, H. Bodenstein, R. Fuchs, M. Hanel, M. Reiser, M. Hensel, M. Clemens, S. Zeynalova, M. Ziepert and M. Pfreundschuh: Equitoxicity of bolus and infusional etoposide: results of a multicenter randomised trial of the German High-Grade Non-Hodgkins Lymphoma Study Group (DSHNHL) in elderly patients with refractory or relapsing aggressive non-Hodgkin lymphoma using the CEMP regimen (cisplatinum, etoposide, mitoxantrone and prednisone). *Ann Hematol* 87, 717-26 (2008)

237. Martinelli G., D. Laszlo, F. Bertolini, R. Pastano, P. Mancuso, A. Calleri, A. Vanazzi, P. Santoro, F. Cavalli and E. Zucca: Chlorambucil in combination with induction and maintenance rituximab is feasible and active in indolent non-Hodgkin's lymphoma. *Br J Haematol* 123, 271-7 (2003)

238. Mey U. J., K. S. Orlopp, D. Flieger, J. W. Strehl, A. D. Ho, M. Hensel, C. Bopp, M. Gorschluter, M. Wilhelm, J. Birkmann, U. Kaiser, A. Neubauer, A. Florschutz, C. Rabe, C. Hahn, A. G. Glasmacher and I. G. Schmidt-Wolf: Dexamethasone, high-dose cytarabine, and cisplatin in combination with rituximab as salvage treatment for patients with relapsed or refractory aggressive non-Hodgkin's lymphoma. *Cancer Invest* 24, 593-600 (2006)

239. Michallet A. S. and B. Coiffier: Recent developments in the treatment of aggressive non-Hodgkin lymphoma. *Blood Rev* 23, 11-23 (2009)

240. Wilson W. H., K. Dunleavy, S. Pittaluga, U. Hegde, N. Grant, S. M. Steinberg, M. Raffeld, M. Gutierrez, B. A. Chabner, L. Staudt, E. S. Jaffe and J. E. Janik: Phase II study of dose-adjusted EPOCH and rituximab in untreated diffuse large B-cell lymphoma with analysis of germinal center and post-germinal center biomarkers. *J Clin Oncol* 26, 2717-24 (2008)

241. Marcus R., K. Imrie, P. Solal-Celigny, J. V. Catalano, A. Dmoszynska, J. C. Raposo, F. C. Offner, J. Gomez-Codina, A. Belch, D. Cunningham, E. Wassner-Fritsch and G. Stein: Phase III study of R-CVP compared with cyclophosphamide, vincristine, and prednisone alone in patients with previously untreated advanced follicular lymphoma. *J Clin Oncol* 26, 4579-86 (2008)

242. Vega M. I., S. Huerta-Yepaz, H. Garban, A. Jazirehi, C. Emmanouilides and B. Bonavida: Rituximab inhibits p38 MAPK activity in 2F7 B NHL and decreases IL-10 transcription: pivotal role of p38 MAPK in drug resistance. *Oncogene* 23, 3530-40 (2004)

243. Jazirehi A. R., S. Huerta-Yepez, G. Cheng and B. Bonavida: Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-{kappa}B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis. *Cancer Res* 65, 264-76 (2005)

244. Jazirehi A. R., M. I. Vega, D. Chatterjee, L. Goodglick and B. Bonavida: Inhibition of the Raf-MEK1/2-ERK1/2 signaling pathway, Bcl-xL down-regulation, and chemosensitization of non-Hodgkin's lymphoma B cells by Rituximab. *Cancer Res* 64, 7117-26 (2004)

245. Vega M. I., A. R. Jazirehi, S. Huerta-Yepez and B. Bonavida: Rituximab-induced inhibition of YY1 and Bcl-xL expression in Ramos non-Hodgkin's lymphoma cell line via inhibition of NF-kappa B activity: role of YY1 and Bcl-xL in Fas resistance and chemoresistance, respectively. *J Immunol* 175, 2174-83 (2005)

246. Wang M., X. H. Han, L. Zhang, J. Yang, J. F. Qian, Y. K. Shi, L. W. Kwak, J. Romaguera and Q. Yi: Bortezomib is synergistic with rituximab and cyclophosphamide in inducing apoptosis of mantle cell lymphoma cells *in vitro* and *in vivo*. *Leukemia* 22, 179-85 (2008)

247. Smolewski P., M. Duechler, A. Linke, B. Cebula, O. Grzybowska-Izydorczyk, M. Shehata and T. Robak: Additive cytotoxic effect of bortezomib in combination with anti-CD20 or anti-CD52 monoclonal antibodies on chronic lymphocytic leukemia cells. *Leuk Res* 30, 1521-9 (2006)

248. de Vos S., A. Goy, S. R. Dakhil, M. N. Saleh, P. McLaughlin, R. Belt, C. R. Flowers, M. Knapp, L. Hart, D. Patel-Donnelly, M. Glenn, S. A. Gregory, C. Holladay, T. Zhang and A. L. Boral: Multicenter randomized phase II study of weekly or twice-weekly bortezomib plus rituximab in patients with relapsed or refractory follicular or marginal-zone B-cell lymphoma. *J Clin Oncol* 27, 5023-30 (2009)

249. Kaufmann H., M. Raderer, S. Wohrer, A. Puspok, A. Bankier, C. Zielinski, A. Chott and J. Drach: Antitumor activity of rituximab plus thalidomide in patients with relapsed/refractory mantle cell lymphoma. *Blood* 104, 2269-71 (2004)

250. Zhang L., Z. Qian, Z. Cai, L. Sun, H. Wang, J. B. Bartlett, Q. Yi and M. Wang: Synergistic antitumor effects of lenalidomide and rituximab on mantle cell lymphoma *in vitro* and *in vivo*. *Am J Hematol* 84, 553-9 (2009)

251. Haritunians T., A. Mori, J. O'Kelly, Q. T. Luong, F. J. Giles and H. P. Koeffler: Antiproliferative activity of RAD001 (everolimus) as a single agent and combined with other agents in mantle cell lymphoma. *Leukemia* 21, 333-9 (2007)

252. Johnson A. J., A. J. Wagner, C. M. Cheney, L. L. Smith, D. M. Lucas, S. K. Guster, M. R. Grever, T. S. Lin and J. C. Byrd: Rituximab and 17-allylamino-17-demethoxygeldanamycin induce synergistic apoptosis in B-cell chronic lymphocytic leukaemia. *Br J Haematol* 139, 837-44 (2007)

253. Sieber S., G. Gdynia, W. Roth, B. Bonavida and T. Efferth: Combination treatment of malignant B cells using the anti-CD20 antibody rituximab and the anti-malarial artesunate. *Int J Oncol* 35, 149-58 (2009)

254. Leonard J. P., J. W. Friedberg, A. Younes, D. Fisher, L. I. Gordon, J. Moore, M. Czuczman, T. Miller, P. Stiff, B. D. Cheson, A. Forero-Torres, N. Chieffo, B. McKinney, D. Finucane and A. Molina: A phase I/II study of galiximab (an anti-CD80 monoclonal antibody) in combination with rituximab for relapsed or refractory, follicular lymphoma. *Ann Oncol* 18, 1216-23 (2007)

255. Zent C. S., T. G. Call, T. D. Shanafelt, R. C. Tschumper, D. F. Jelinek, D. A. Bowen, C. R. Secreto, B. R. Laplant, B. F. Kabat and N. E. Kay: Early treatment of high-risk chronic lymphocytic leukemia with alemtuzumab and rituximab. *Cancer* 113, 2110-8 (2008)

256. Ganjoo K. N., C. S. An, M. J. Robertson, L. I. Gordon, J. A. Sen, J. Weisenbach, S. Li, E. A. Weller, A. Orazi and S. J. Horning: Rituximab, bevacizumab and CHOP (RA-CHOP) in untreated diffuse large B-cell lymphoma: safety, biomarker and pharmacokinetic analysis. *Leuk Lymphoma* 47, 998-1005 (2006)

257. Leonard J. P., S. J. Schuster, C. Emmanouilides, F. Couture, N. Teoh, W. A. Wegener, M. Coleman and D. M. Goldenberg: Durable complete responses from therapy with combined epratuzumab and rituximab: final results from an international multicenter, phase 2 study in recurrent, indolent, non-Hodgkin lymphoma. *Cancer* 113, 2714-23 (2008)

258. Dijoseph J. F., M. M. Dougher, D. C. Armellino, L. Kalyandrug, A. Kunz, E. R. Boghaert, P. R. Hamann and N. K. Damle: CD20-specific antibody-targeted chemotherapy of non-Hodgkin's B-cell lymphoma using calicheamicin-conjugated rituximab. *Cancer Immunol Immunother* 56, 1107-17 (2007)

259. Niwa R., M. Sakurada, Y. Kobayashi, A. Uehara, K. Matsushima, R. Ueda, K. Nakamura and K. Shitara: Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. *Clin Cancer Res* 11, 2327-36 (2005)

260. Li B., L. Zhao, H. Guo, C. Wang, X. Zhang, L. Wu, L. Chen, Q. Tong, W. Qian, H. Wang and Y. Guo: Characterization of a rituximab variant with potent antitumor activity against rituximab-resistant B-cell lymphoma. *Blood* 114, 5007-15 (2009)

261. Bingaman M. G., G. D. Basu, T. C. Golding, S. K. Chong, A. J. Lassen, T. J. Kindt and C. A. Lipinski: The autophilic anti-CD20 antibody DXL625 displays enhanced potency due to lipid raft-dependent induction of apoptosis. *Anticancer Drugs* 21, 532-42 (2010)

262. Rossi E. A., D. M. Goldenberg, T. M. Cardillo, R. Stein, Y. Wang and C. H. Chang: Novel designs of multivalent anti-CD20 humanized antibodies as improved lymphoma therapeutics. *Cancer Res* 68, 8384-92 (2008)

263. Li B., S. Shi, W. Qian, L. Zhao, D. Zhang, S. Hou, L. Zheng, J. Dai, J. Zhao, H. Wang and Y. Guo: Development of novel tetravalent anti-CD20 antibodies with potent antitumor activity. *Cancer Res* 68, 2400-8 (2008)

264. Rossi E. A., D. M. Goldenberg, T. M. Cardillo, R. Stein and C. H. Chang: Hexavalent bispecific antibodies represent a new class of anticancer therapeutics: 1. Properties of anti-CD20/CD22 antibodies in lymphoma. *Blood* 113, 6161-71 (2009)

265. James S. E., N. N. Orgun, T. F. Tedder, M. J. Shlomchik, M. C. Jensen, Y. Lin, P. D. Greenberg and O. W. Press: Antibody-mediated B-cell depletion before adoptive immunotherapy with T cells expressing CD20-specific chimeric T-cell receptors facilitates eradication of leukemia in immunocompetent mice. *Blood* 114, 5454-63 (2009)

Key Words: CD20, Monoclonal Antibodies, Rituximab, Tositumomab, Ofatumumab, Non Hodgkin Lymphoma, Chronic Lymphocytic Leukemia, Complement, Antibody-Dependent Cellular Cytotoxicity, Apoptosis, Review

Send correspondence to: Jakub Golab, Department of Immunology, Center of Biostructure Research, The Medical University of Warsaw, 1a Banacha Str., F building, 02-097 Warsaw, Tel:48-22-599-2198, Fax: 48-22-599-2194, E-mail: jakub.golab@wum.edu.pl