REVIEW ARTICLE

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The regulation and function of CD20: an "enigma" of B-cell biology and targeted therapy

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ABSTRACT

The introduction of anti-CD20 monoclonal antibodies such as rituximab, ofatumumab, or obinutuzumab improved the therapy of B-cell malignancies even though the precise physiological role and regulation of CD20 remains unclear. Furthermore, CD20 expression is highly variable between different B-cell malignancies, patients with the same malignancy, and even between intraclonal subpopulations in an individual patient. Several epigenetic (EZH2, HDAC1/2, HDAC1/4, HDAC6, complex Sin3A-HDAC1) and transcription factors (USF, OCT1/2, PU.1, PiP, ELK1, ETS1, SP1, NFκB, FOXO1, CREM, SMAD2/3) regulating CD20 expression (encoded by *MS4A1*) have been characterized. CD20 is induced in the context of microenvironmental interactions by CXCR4/SDF1 (CXCL12) chemokine signaling and the molecular function of CD20 has been linked to the signaling propensity of B-cell receptor (BCR). CD20 has also been shown to interact with multiple other surface proteins on B cells (such as CD40, MHCII, CD53, CD81, CD82, and CBP). Current efforts to combine anti-CD20 monoclonal antibodies with BCR signaling inhibitors targeting BTK or PI3K (ibrutinib, acalabrutinib, idelalisib, duvelisib) or BH3-mimetics (venetoclax) lead to the necessity to better understand both the mechanisms of regulation and the biological functions of CD20. This is underscored by the observation that CD20 is decreased in response to the "BCR inhibitor" ibrutinib which largely prevents its successful combination with rituximab. Several small molecules (such as histone deacetylase inhibitors, DNA methyl-transferase inhibitors, aurora kinase A/B inhibitors, farnesyltransferase inhibitors, FOXO1 inhibitors, and bryostatin-1) are being tested to upregulate cell-surface CD20 levels and increase the efficacy of anti-CD20 monoclonal antibodies. Herein, we review the current understanding of CD20 function, and the mechanisms of its regulation in normal and malignant B cells, highlighting the therapeutic implications.

Introduction

The approval of the anti-CD20 antibody rituximab by the Food and Drug Administration in 1997 was a conceptual breakthrough in the treatment of B-cell malignancies. Rituximab improved progression-free survival and overall survival rates when added to chemotherapy in "mature" B-cell leukemias and lymphomas such as chronic lymphocytic leukemia (CLL), follicular lymphoma, and diffuse large B-cell lymphoma (DLBCL), and this proved that monoclonal antibodies could be used in cancer treatment.[1] Additionally, rituximab maintenance therapy has been introduced for some of these diseases. Based on the success of rituximab, new engineered anti-CD20 monoclonal antibodies, namely ofatumumab and obinutuzumab, were developed. Preclinical studies suggest that these new anti-CD20 monoclonal antibodies are superior to rituximab for some mechanisms of action.[2] Anti-CD20 monoclonal antibodies might act through several mechanisms (Figure 1) including complement-dependent cytotoxicity (CDC), complement-dependent cellular cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), antibody-

dependent cellular phagocytosis, and direct apoptosis induction, as has been elegantly reviewed by others.[3,4] All these mechanisms were observed *in vitro* and/or in animal models, and likely act *in vivo* in patients as well, but their relative contribution to the clinical effects of the different anti-CD20 monoclonal antibodies is still debated. It is also unclear why the application of novel engineered monoclonal antibodies provides clinical benefit in comparison to rituximab in some B-cell malignancies, but not in others. For example in CLL patients, obinutuzumab is superior to rituximab when combined with chlorambucil, as judged by the number of complete remissions and prolonged progression-free survival.[5] A much less significant improvement in progression-free survival has also been demonstrated in previously untreated follicular lymphoma patients treated with obinutuzumab-based chemoimmunotherapy compared to rituximab-based chemoimmunotherapy.[6,7] Finally, a phase III clinical study demonstrated no improvement in progression-free survival in a large cohort of treatment-naïve DLBCL patients when comparing obinutuzumab plus CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) *versus* rituximab plus CHOP.[8] It is important to note that in these trials, obinutuzumab was used at doses and schedules quite different from those of rituximab. For example, in the CLL trial[5] a flat obinutuzumab dose of 1000 mg/patient was used (on days 1, 8, and 15 of cycle 1 and on day 1 of cycles 2-6), while rituximab was used at a dose of 375 mg/m^2 on day 1 of cycle 1 and 500 mg/m^2 on day 1 of cycles 2-6. Overall, in this CLL trial the median cumulative rituximab dose per patient was 64% of the obinutuzumab dose (these two monoclonal antibodies have a nearly identical molecular weight).

Currently, efforts have shifted from adding anti-CD20 monoclonal antibodies to chemotherapy to combining them with novel drugs, such as B-cell receptor (BCR) signaling inhibitors (ibrutinib, idelalisib, etc.)[9] or BH3-mimetics inhibiting BCL2 (venetoclax),[10] and also the development of CD20 targeting chimeric antigen receptor T cells.[11] It is essential to understand the mechanism of CD20 regulation and function thoroughly and to elucidate the mechanism of action of monoclonal antibodies in

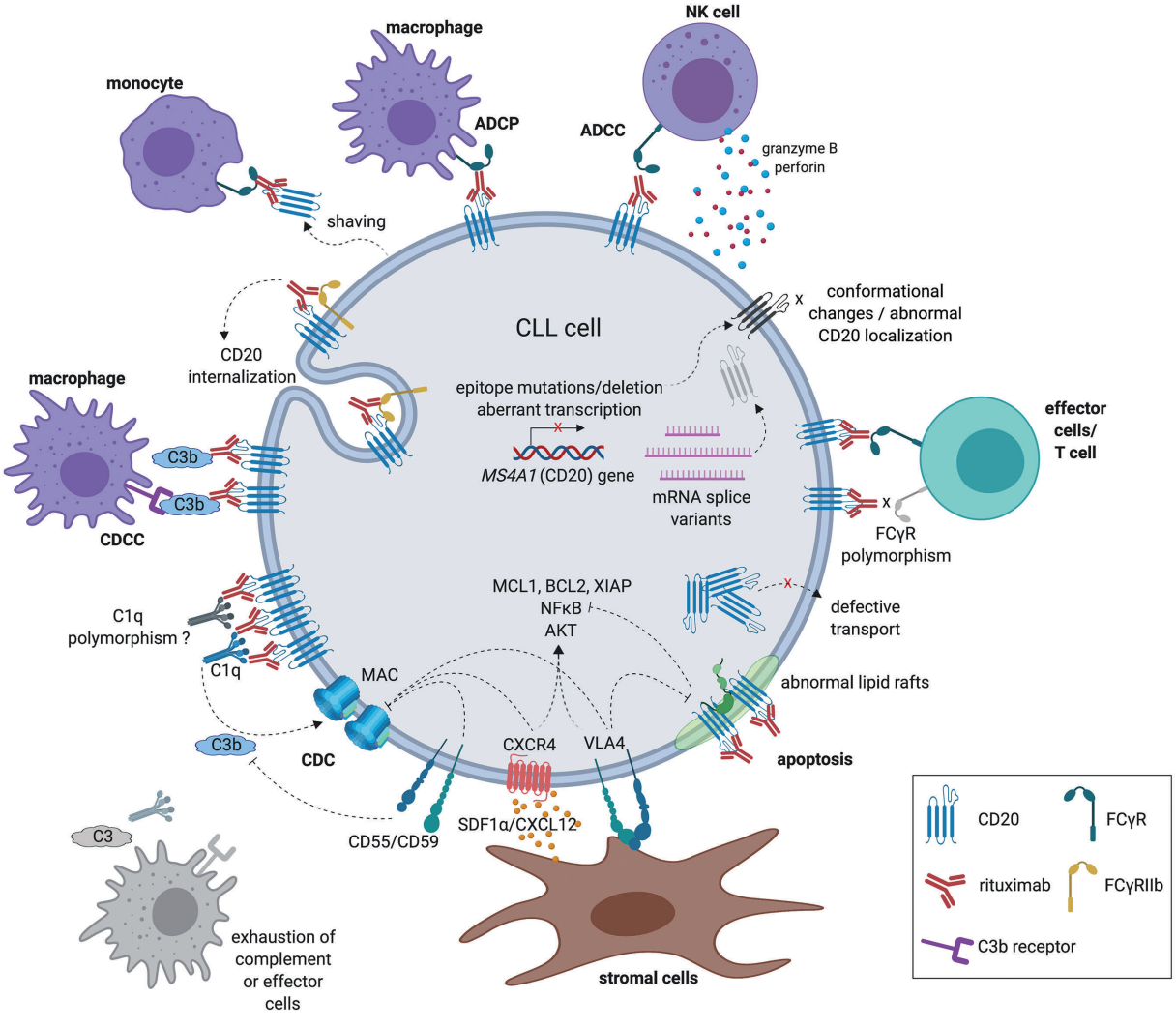

Figure 1. Summary of the known mechanisms of action of anti-CD20 monoclonal antibodies and an overview of potential factors affecting resistance to anti-CD20 therapy in malignant B cells. Anti-CD20 monoclonal antibodies act through several mechanisms, including complement-dependent cytotoxicity (CDC), complement-dependent cellular cytotoxicity (CDCC), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and induction of direct apoptosis.

order to fully exploit their therapeutic potential. This is underscored by the recent disappointing results of clinical trials testing rituximab's addition to the BTK inhibitor ibrutinib in CLL, which showed practically no benefit of such a combination.[12] Here we summarize the research describing the regulation and function of CD20 in normal and malignant B cells, and the therapeutic implications of these observations, including the relevance for the combination of "BCR inhibitors" with anti-CD20 monoclonal antibodies.

CD20 gene and protein structure

CD20 is a 33-37 kDa non-glycosylated protein expressed on the surface of normal and malignant B lymphocytes, and belongs to the MS4A (membrane-spanning 4-domain family A) protein family.[13] To date, 18 MS4A family members have been identified, besides *MS4A1* (encoding CD20), also the high-affinity immunoglobulin E receptor β subunit (MS4A2/FcεRIβ) or *HtM4* gene (MS4A3) (reviewed by Eon Kuek[14]). MS4A proteins are transmembrane molecules and they are predicted to share a similar polypeptide sequence and overall topological structure. The majority of *MS4A* genes, including *MS4A1*, are localized within a cluster on chromosome 11q12 in humans (chromosome 19 in mice), and two members from a closely related *TMEM176* gene family were identified in chromosome region 7q36.1.[14]

The *MS4A1* gene is 16 kb long, comprises eight exons, and several different CD20 mRNA transcripts have been annotated.[13] The dominant CD20 mRNA variant is 2.8 kb long and uses all eight exons, whereas the second most common form is 263 bases shorter, as it skips exon II. A minor 3.5 kb mRNA results from splicing exons in the upstream region into an internal 3' splice site located in exon I. However, all three transcripts are translated into identical full-length CD20 protein as the translation start codon is localized within exon III. Moreover, other alternative transcripts were identified in malignant B cells, some of them encoding truncated forms of CD20 protein leading to impaired binding of anti-CD20 monoclonal antibodies.[15,16]

CD20 protein consists of four hydrophobic transmembrane domains, one intracellular and two extracellular domains (large and small loops) with both N- and C- termini residing within the cytosol.[14] Three CD20 isoforms (33, 35 and 37 kDa) resulting from different phosphorylation have been identified, and CD20 phosphorylation was reported to be higher in proliferating malignant B cells than in resting B cells.[17] Normally, CD20 does not form hetero-oligomers,[18] but exists on the cell surface as homo-

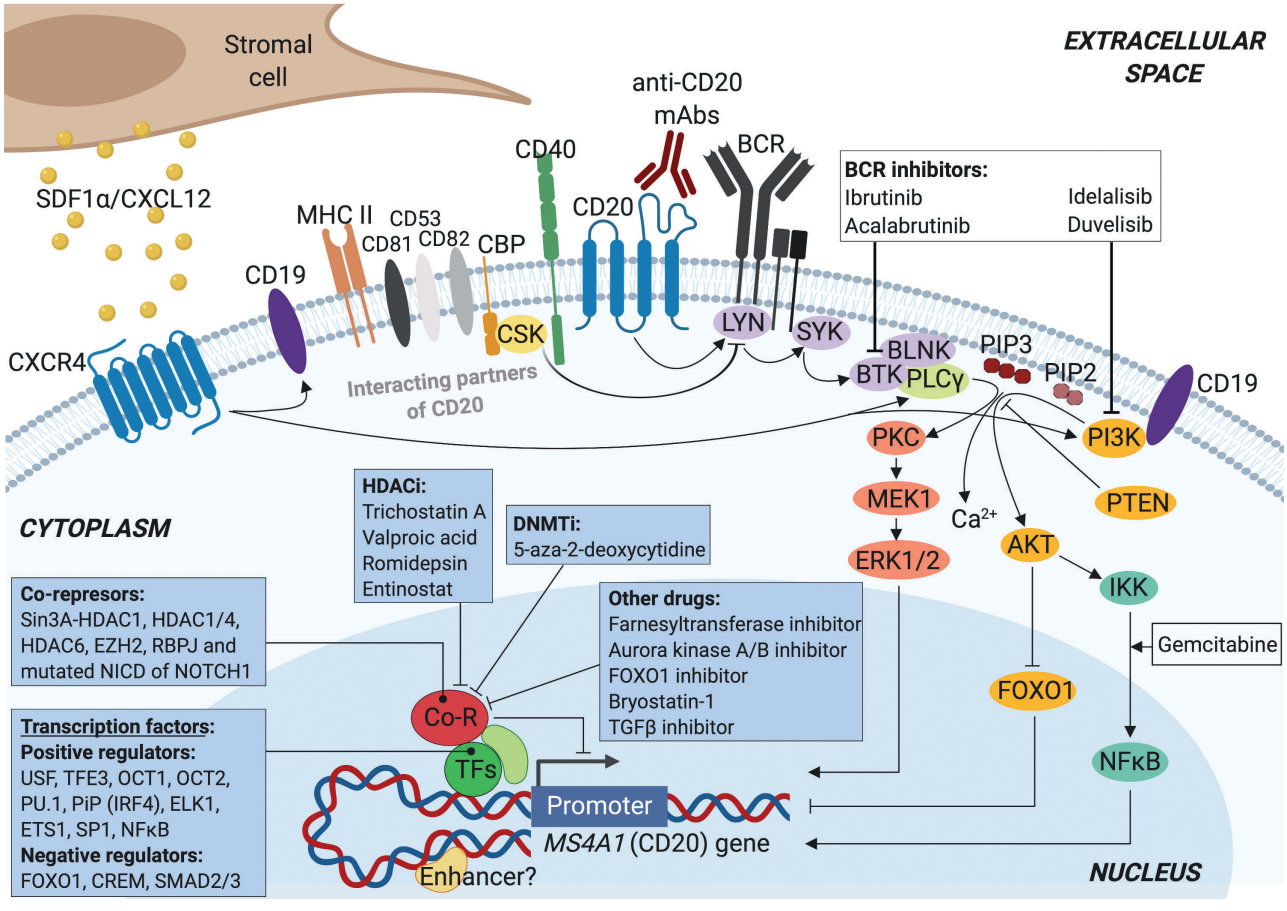

Figure 2. A schematic view of interacting partners of CD20 on cell membrane and mechanisms of CD20 gene (*MS4A1*) regulation in malignant B cells. mAbs: monoclonal antibodies; BCR: B-cell receptor; HDACi: histone deacetylase inhibitors; DNMTi: DNA methyl-transferase inhibitors; NICD: NOTCH1 intracellular domain; TGFβ: transforming growth factor β.

dimeric and homo-tetrameric oligomers associated with other cell-surface and cytoplasmic proteins contributing to the signal transduction.[17,19,20] Tetraspanin proteins tend to associate with multiple other proteins in membrane microdomains (Figure 2).[21] Energy transfer experiments indicate that CD20 is in close proximity to other tetraspan molecules, such as CD53, CD81, and CD82, forming supramolecular complexes (Figure 2).[22] CD20 is also known to be physically coupled to major histocompatibility complex class II (MHCII), CD40 molecule, BCR, and the C-terminal src kinase-binding protein (CBP) that interacts with Src kinases such as LYN, FYN, and LCK (Figure 2).[20,23,24] Besides the transmembrane form of CD20, circulating CD20 was reported in CLL patients' plasma;[25] however, this is likely to be part of a larger protein complex or a cell membrane fragment originating from cell breakdown.

CD20 is a general B-cell marker expressed by the majority of B cells starting from late pre-B lymphocytes (it is not expressed by pro-B lymphocytes), and its expression is lost in terminally differentiated plasmablasts and plasma cells. Recently, a subset of $CD20^+$ T cells with immune-regulatory and pro-inflammatory activity has been described; however, the clinical relevance of this remains to be determined.[26] In B-cell malignancies, the level of CD20 expression is extremely variable depending on the specific neoplasm, with the lowest CD20 expression usually being observed in patients with CLL and the highest CD20 cell-surface expression on DLBCL and hairy cell leukemia cells.[27,28] Within CLL, it was noted that CD20 expression was also relatively higher in a disease subtype with a mutated variable region of immunoglobulin gene (IGHV) than in the subtype with unmutated IGHV.[29] Some studies described that higher CD20 expression levels correlate with longer overall survival in patients with B-cell lymphomas treated with rituximab,[30,31] although this remains controversial.[32,33] Notably, CD20 levels are heterogeneous not only among patients with the same malignancy, but also within the intraclonal cell subpopulations in an individual patient.[34]

CD20 function: a link to B-cell receptor signaling and microenvironmental interactions

The biological function of CD20 in B cells and its physiological ligand, if any, remain unclear. Some light on CD20 function has been shed by a case report of a patient with a common variable immunodeficiency and CD20 loss caused by a homozygous mutation in an exon 5 splicing site of *MS4A1*. The mutation led to alternative splicing with complete deletion of exon 5 and insertion of intron sequences and thus a truncated form of *MS4A1* mRNA.[35] Due to this homozygous mutation, the patient completely lacked cell-surface CD20. This did not disturb precursor B-cell differentiation in the bone marrow, as the patient had normal serum IgM levels and normal B-cell numbers. However, CD20 deficiency resulted in a reduced number of circulating memory B cells, reduced isotype switching of Ig, and decreased IgG antibody levels. In agreement with this observation, challenging the patient's primary B cells *in vitro* using T-dependent and T-independent antigens led to the normal proliferation and secretion of IgM but reduced production of IgG. Given these data it is surprising that after repeated vaccinations the patient displayed a reduced ability to respond to T-independent antigens (pneumococcal polysaccharide vaccine), but a normal reaction to T-dependent antigens (anti-tetanus toxoid IgG).

Cases of a homozygous mutation in the *MS4A1* gene in humans are extremely rare, which prompted the generation of mouse models. This is a reasonable approach, since human and mice CD20 proteins share most structural features and a conserved amino acid sequence (~75% homology) with only a few structural modifications in the transmembrane and N- and C-terminal cytoplasmic domains.[36] CD20 in both humans and mice is B-cell specific, being first expressed by late pre-B cells in the bone marrow, predominantly after Ig heavy chain rearrangement. Uchida *et al.* created a mouse model with a homozygous mutation in the *MS4A1* gene.[36] These CD20-less mice had normal B-cell differentiation, isotype switching, maturation, mitogen-induced proliferation, and tissue localization. Similarly, CD20 deletion was not observed to have any effect on proliferation and differentiation in mice with *MS4A1* disruption, generated by Neuberger's group.[37] $CD20^{-/-}$ mice immunized with T-dependent antigens showed impaired humoral immunity and primary and secondary immune responses connected with reduced numbers of germinal center B cells.[38] Altogether, these studies in human and murine CD20-deficient B cells suggest that CD20 is required for both optimal T-independent humoral immunity, and also for a response to T-dependent antigens. However, it should be taken into consideration that the T-dependent immune response might be impaired due to the loss of CD20 in a small $CD20^+$ population of T cells whose specific role in the immune system remains unclear.[26] Overall, the relatively mild phenotype resulting from CD20 loss in humans and mice is somewhat surprising since CD20 was reported to be physically and functionally coupled to MHCII and CD40 (Figure 2),[23] which are both critical for B- and T-cell interactions.

The development of humoral immunity requires a functional BCR signaling pathway, and CD20 was reported to be co-localized in lipid rafts[39] and to interact directly physically with BCR.[20] Additionally, it has been observed that CD20 becomes heavily phosphorylated after mitogen stimulation, and it has been proposed that it might function as a calcium channel and be involved in B-cell activation.[17,19] This is in line with *in vitro* data showing that BCR-activated calcium flux was reduced after siRNA-mediated CD20 down-modulation in human B-cell lines.[34,40] Moreover, direct CD20 crosslinking induces acute signaling similar to BCR crosslinking, including calcium flux, and overlapping transcription patterns in human lymphoma cell lines.[41,42] Kheirallah *et al.* also demonstrated that pretreatment of lymphoma cell lines with rituximab interferes with BCR signaling cascade stimulation, suggesting that both cell-surface proteins might share the same signaling pathway components and activate negative feedback regulatory mechanisms, including BCR downmodulation.[43] We and others have shown that levels of cell-surface CD20 on primary CLL cells are correlated (and possibly co-regulated) with cell-surface BCR expression.[34] Additionally, we observed *in vivo* that CLL cells that have recently exited the lymph node microenvironment to the peripheral blood are characterized by a marked upregulation of CD20 levels.[44] This stems from the activation of CXCR4 by SDF1 chemokine, which leads to transcriptional activation of CD20 expression. Moreover, CD20 cell-surface levels are

induced in CLL cells treated by microenvironmental factors such as IL4, TNFα, INFα or GMCSF *in vitro*[45-47](and our unpublished data).

CD20 silencing in malignant B cells revealed that CD20 affects the phosphorylation of multiple BCR-associated kinases and proteins after BCR-ligation (LYN, SYK, GAB1, and ERK).[34] This suggests that both CD20 and BCR are induced in immune niches[34] to allow effective and strong BCR activation by an antigen or CD20 might also be involved in some form of "tonic" BCR signaling.[48] This has important implications for combining BCR inhibitors with antibodies targeting CD20. We have shown that inhibiting BTK interferes with CXCR4 signaling in CLL cells and thus leads to very significant repression of CD20 expression in CLL cells. This might partially explain the lack of clinical benefit from adding rituximab to ibrutinib.[12] Ibrutinib was recently tested and approved in combination with a more potent anti-CD20 monoclonal antibody, namely obinutuzumab,[49] whose efficacy is less affected by lower levels of CD20 on the cell-surface. We also suggest that PI3K inhibition, like BTK inhibition, might lead to the downmodulation of CD20, but this remains to be formally proven, and the implications for the therapeutic combination of rituximab with PI3K inhibitors (including idelalisib) or other BTK inhibitors (such as acalabrutinib) are unclear.

Altogether, functional studies suggest that CD20 is physiologically directly required for efficient BCR signaling in B cells. This is also in line with some data from the CD20 mouse models. In Uchida's CD20$^{-/-}$ mice model, cell-surface IgM expression on both mature and immature B cells was 20 – 30% lower than that on B cells from wild-type littermates, which was connected with reduced BCR- and CD19-dependent intracellular calcium mobilization.[36] In a study by Morsy *et al.*, the reduction in BCR-associated calcium mobilization in CD20$^{-/-}$ murine B cells was proposed to be caused by a defect in calcium transport rather than in its release from intracellular stores.[38] In our opinion, there is a sufficient body of evidence suggesting that CD20 is involved in BCR signaling, but it is unclear whether this is related to its putative function as a calcium channel and/or other function(s). Similarly, it is not clear if other molecular pathways or B-T-cell interactions might be affected by CD20 levels on the cell-surface of B cells.

Regulation of CD20 transcription and its "therapeutic modulation"

Rituximab is one of the most effective and widely used therapeutic monoclonal antibodies, but malignant B cells can become relatively resistant to such therapy. Mechanisms of malignant B cells' resistance to anti-CD20 monoclonal antibodies include insufficient CDC activity due to increased expression of regulatory proteins CD55, CD59 or factor H,[50,51] less effective ADCC in cases with specific FcγRIII polymorphism,[52] exhaustion of cytotoxic mechanisms (such as complement/effector cells),[53,54] polymorphism in the complement component C1qA,[55] or abnormal composition and localization of lipid rafts and thus impaired rituximab-induced apoptosis (Figure 1).[56] Nevertheless, one of the most straightforward and frequent causes of resistance to anti-CD20 monoclonal antibodies is reduced CD20 expression, which can be due to (de)regulation of transcriptional, post-transcriptional, or post-translational mechanisms (including CD20 protein transport to the cell surface[57]).

Regarding transcriptional regulation, the *MS4A1* gene lacks several regulatory elements typical of other B-cell specific genes, including TATA and CAAT box. The

Table 1. Positive and negative regulators of *MS4A1* (CD20 gene) transcription.

	Positive regulators[Ref]	Negative regulators[Ref]
Transcription factors		
	USF, TFE3[59]	FOXO1[70]
	OCT1,[58] OCT2[58]	CREM[69]
	PU.1/PiP (IRF4)[59]	SMAD2/3[99]
	ELK1,[64] ETS1[64]	MYC[67]
	SP1[65]	
	NFκB[65]	
	CHD4,[69]* MBD2[69]*	
Epigenetic regulators		
	Unknown	Sin3A-HDAC1[76]
		EZH2[80]
		HDAC1/4[81]
		HDAC6[82]
		RBPJ and mutated NICD of NOTCH1[95]

* The DNA binding site in the *MS4A1* promoter has not been defined. NICD: NOTCH1 intracellular domain.

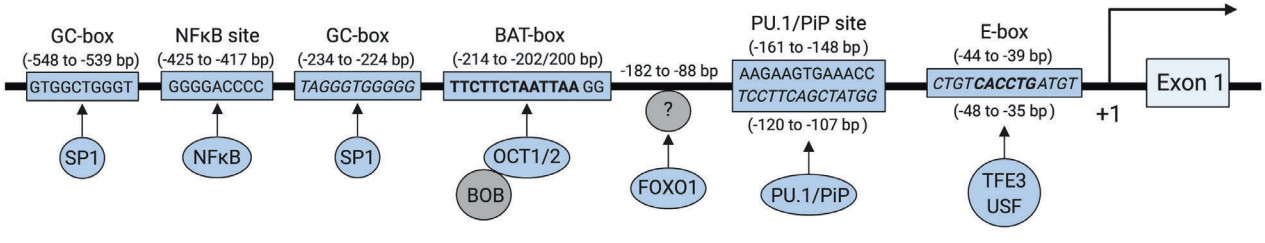

Figure 3. A schematic of the proximal region of *MS4A1* promoter with transcription factor binding sites. Several regulatory elements differ in nucleotide sequence when comparing data from literature and the TRANSFAC database. Shimizu *et al.*[65] described the GC-box binding SP1 as being located in a region between bases -548 and -539, but the TRANSFAC database identified a GC-box in a position -234 to -224 bp [based on the SP1 chromatin immunoprecipitation sequencing data (ENCODE ID: ENCSR000BHK)]. A BAT-box was identified by Thévenin *et al.*[58] in the proximal promoter region located between bases -214 and -202 (in bold), while others describe it between bases -214 and -200.[59,65] The PU.1/PiP binding site was originally defined as a sequence between -161 and -148 bp,[59] but TRANSFAC predicts it in the region from -120 to -107 bp (in italics). The E-box sequence is usually described as the CACCTG sequence between -44 and -39 bp (in bold)[59,62,65] but the TRANSFAC database suggests (based on ENCODE ID: ENCSR000BGI) a longer sequence from -48 to -35 bp (in italics). FOXO1 was suggested as being recruited to the *MS4A1* promoter indirectly by the DNA-binding element between bases -182 and -88.[70] NFκB binds into the region of the *MS4A1* promoter between -425 and -417 bp.[62]

known positive regulatory elements present in the *MS4A1* promoter include an E-box motif (binding μE3-specific transcription factors such as USF and TFE3), "PU.1/PiP" binding site and a BAT box (Figure 3, Table 1).[58,59] The BAT box is a sequence element present in the most proximal region and serves as a binding site for the transcription factors OCT1 and OCT2 with a B-cell restricted co-activator BOB (Figure 3, Table 1).[58,60] The BAT element is important for the high constitutive expression of CD20 in mature B cells and the induction of CD20 in pre-B cells.[58] The "PU.1/PiP" binding site is a putative site for transcription factors belonging to the ETS family (e.g. PU.1) and protein PiP (IRF4) (Figure 3, Table 1). PiP is recruited to this DNA binding site indirectly by phosphorylated PU.1[59] and a "PU.1/PiP" binding site seems to be critical for CD20 expression as it is occupied only in CD20-positive B cells. Additionally, PU.1/Pip are downregulated during plasma cell differentiation,[61] and mutations in this binding site nearly completely abolished the promoter activity of *MS4A1*.[59] Moreover, transcriptional CD20 activation in primary CLL and non-Hodgkin lymphoma (NHL) B cells was associated with increased PU.1 and OCT2 binding to the *MS4A1* promoter in response to farnesyltransferase inhibition.[62] Downregulating PU.1 expression by overexpression of its negative regulator, namely FLT3, also led to lower CD20 expression in CLL cells and *vice versa*.[63]

Several transcription factors from the ETS family, such as ELK1 and ETS1, were observed to be activated in an ERK-dependent manner and enhance CD20 cell-surface expression in B-NHL cell lines and primary CLL cells after bryostatin-1 treatment *in vitro* (which activates the MEK1/ERK-1/2 pathway via PKC) (Figure 2, Table 1).[64] Furthermore, it was proposed that NFκB might positively regulate CD20 expression[62,65] and gemcitabine treatment of DLBCL cell lines augmented CD20 expression together with NFκB signaling activation (Figures 2 and 3, Table 1).[66] Chromatin immunoprecipitation sequencing analysis also revealed *MS4A1* as a direct MYC target gene in Burkitt lymphoma cell lines (Table 1), and MYC silencing resulted in CD20 upregulation.[67] The repression of CD20 by MYC is surprising and remains to be confirmed in other lymphoma cell types, since B-cell activation (also leading to MYC expression) is generally known to induce CD20 expression in B cells.[68] To reveal other factors regulating CD20 expression, Slabicki *et al.*[69] performed a genome-wide RNA interference screening using a library of small hairpin RNAs delivered into Raji cells (Burkitt lymphoma cell line) by lentiviral vectors. They identified 37 potential CD20 repressors and 51 activators, among them CHD4 and MBD2 as novel *MS4A1* inducers (Table 1). Both CHD4 and MBD2 are members of the nucleosome remodeling deacetylase complex, which plays an important role in the regulation of gene transcription. This screening also revealed CREM as the top candidate for CD20 repression, and the presence of three half-cAMP response elements in *MS4A1* promoter sites (TGACG) led to the notion that cAMP-mediated signal transduction plays a role in CD20 transcriptional repression. Most recently, FOXO1 transcription factor was described as a negative *MS4A1* transcription regulator in lymphoma B cells (Figures 2 and 3, Table 1).[70] This is in agreement with the observation that DLBCL patients with activating FOXO1 mutations have shorter overall survival upon rituximab-based therapy.[71] As the exact localization of the putative FOXO1 binding site in the *MS4A1* promoter was not determined, it is believed that FOXO1 binds indirectly to the DNA-binding element between -182 and -88 bp (Figure 3).[70] These data (and our unpublished data) suggest that FOXO1 inhibitors might theoretically be combined with anti-CD20 antibodies to induce CD20 expression and potentiate the effect of the monoclonal antibodies. Similarly, other groups have proposed that inhibiting aurora kinase A/B could also lead to upregulation of CD20 and potentiation of rituximab's clinical efficacy.[72,73]

It is not surprising that several recent studies suggested that CD20 is at least partially regulated by epigenetic mechanisms. Tomita *et al.* demonstrated that treating a CD20-negative B-cell line with the histone deacetylase (HDAC) inhibitor trichostatin A resulted in robust upregulation of CD20 mRNA and protein.[74] *In vitro* treatment of primary cells obtained from relapsed CD20-negative B-NHL patients using the DNA methyl-transferase (DNMT) inhibitor 5-aza-2-deoxycytidine also led to the stimulation of *MS4A1* mRNA and cell-surface expression within 3 days, and restoration of rituximab sensitivity.[75] Despite the fact that CD20 stimulation by DNMT inhibitors was described both *in vitro*[75,76] and *in vivo* in patients with B-cell malignancies,[77] CD20 is less likely to be regulated by CpG (de)methylation as its promoter region does not contain any CpG islands up to ~5 kb upstream from the transcription start site.[76] However, it is plausible that DNMT inhibition regulates the methylation status of transcription factors critical for *MS4A1* transcription, or some more distant genomic regions (enhancers) are involved in *MS4A1* transcription. Furthermore, it was reported that a Sin3A-HDAC1 co-repressor complex is recruited to the *MSA41* promoter in CD20-negative B-cell lines (Figure 2, Table 1).[76] This complex dissociates from the promoter with 5-aza-2-deoxycytidine and trichostatin A treatment, resulting in histone acetylation and partial restoration of CD20 expression. Shimizu *et al.* showed that HDAC inhibitors (valproic acid or romidepsin) are able to induce CD20 expression in B-cell lines through *MS4A1* promoter hyperacetylation and recruit the SP1 transcription factor within 48 hours (Figures 2 and 3, Table 1).[65] At the moment, several ongoing clinical trials are evaluating the efficacy of epigenetic modulators in combination with rituximab (Table 2). In the VALFRID study, pretreatment with valproic acid before first-line therapy with CHOP plus rituximab in DLBCL patients resulted in histone acetylation, CD20 upregulation at the mRNA and cell-surface levels[78] and improved overall survival.[79] In contrast, the analysis of three CLL patients from the PREVAIL study showed no CD20 induction upon pretreatment with valproic acid.[80] A plausible explanation might be that valproic acid induces a bivalent *MS4A1* promoter status in primary CLL cells *in vivo* as it induces histone acetylation, but also transient recruitment of the transcriptional repressor EZH2 to the *MS4A1* promoter (Figure 2, Table 1). Administering a DNMT inhibitor and pan-HDAC inhibitor (valproic acid, romidepsin, trichostatin A, SAHA) stimulates CD20 expression and might improve anti-CD20 therapy *in vivo*, at least in some patients with B-NHL. However, the clinical use of pan-HDAC inhibitors is hindered by adverse effects[77,79] and thus the involvement of individual HDAC molecules and selective HDAC inhibitors are undergoing pre-clinical studies. Recently, entinostat, a selective HDAC1/4 inhibitor, was

Table 2. List of novel drugs combined with anti-CD20 monoclonal antibodies in B-cell malignancies.

	Drugs	Pre-clinical studies	Phase I: trial ID* and status	Phase II: trial ID* and status	Phase III: trial ID* and status	FDA approvals and comments
BCR inhibitors	Ibrutinib	↓CD20 in CLL cells *in vitro* and *in vivo*[44,100]		NCT02007044 (active)[12]	NCT02264574 (completed)[49] NCT02165397 (active)[118]	FDA approved (2019) ibrutinib plus obinutuzumab for treatment-naïve CLL. No benefit from combination of ibrutinib and rituximab in CLL (NCT02007044).[12] FDA approved (2018) ibrutinib in combination with rituximab for Waldenström's macroglobulinemia.
	Idelalisib	Unknown			NCT01539512 (completed)[110] NCT01659021 (terminated)[111]	FDA approved (2014) idelalisib in combination with rituximab for relapsed CLL.
	Duvelisib	Unknown		NCT02391545 (terminated)	NCT02204982 (terminated)	-
	Acalabrutinib	BTK inhibition ↓CD20 in CLL cells *in vitro* and *in vivo*[44,100]			NCT02475681 (active)[109]	-
BCL2 inhibitor	Venetoclax	Unknown	NCT02296918 (active)[119]		NCT02950051 (active) NCT02005471 (active)[10] NCT02242942 (active)[116]	FDA approved (2018) venetoclax in combination with rituximab for CLL/SLL patients, with or without 17p deletion, who have received at least one prior therapy. FDA approved (2019) venetoclax in combination with obinutuzumab for previously untreated CLL/SLL.
Chromatin modulators	Valproic acid	↑CD20 in B-cell lines *in vitro*[65] and in DLBCL patients *in vivo*[78]	NCT01622439 (completed)[79] NCT02144623 (completed)[80]			
	5-Azacitidine, 5-aza-2-deoxycytidine	↑CD20 *in vitro*[75,76] and *in vivo*[77]	NCT01004991 (completed) NCT00901069 (completed)			
	Trichostatin A	↑CD20 in B-cell lines *in vitro*[74]				
	Romidepsin	↑CD20 in B-cell lines *in vitro*[65]				
	Entinostat	↑CD20 in B-cell lines *in vitro*[81]				
Other drugs	Bryostatin-1	↑CD20 in B-cell lines *in vitro*[64]			NCT00087425 (completed)	
	Gemcitabine	↑CD20 in B-cell lines *in vitro*[66]			NCT00169195 (completed)[120] NCT02750670 (active)	
	CpG oligodeoxynucleotides	↑CD20 in CLL cells *in vitro*[121]			NCT00251394 (completed)[122]	
	Aurora kinase A/B inhibitor (alisertib)	↑CD20 in immunotherapy-resistant cell lines *in vitro*[123]	NCT01397825 (completed)[72] NCT01695941 (active)			
	Farnesyltransferase inhibitor (L-744, 832)	↑CD20 in B-cell lines *in vitro*[62]				
	TGFβ inhibitor (LY364947)	inhibits the suppression of CD20 mediated by TGFβ *in vitro*[99]				
	FOXO1 inhibitors (AS1842856)	↑CD20 expression *in vitro* and *in vivo*[70]				

↑: induction of expression; ↓: repression of expression; -: no decision; *: ID on clinicaltrials.gov.; ID: identity; FDA: Food and Drug Administration; CLL: chronic lymphocytic leukemia; BCR: B-cell receptor; BTK: Bruton tyrosine kinase; SLL: small lymphocytic lymphoma; DLBCL: diffuse large B-cell lymphoma; TGFβ: transforming growth factorβ.

reported to upregulate CD20 and improve rituximab efficacy both *in vitro* and in a mouse model (Figure 2, Table 1).[81] Additionally, Bobrowicz *et al.* identified HDAC6 as a novel repressor of CD20 expression in B-cell lines and primary CLL cells (Figure 2, Table 1).[82] HDAC6 was shown to be overexpressed in CLL cells and its inhibition augmented the efficacy of anti-CD20 monoclonal antibodies *in vitro* and improved survival of complement-, NK cell- and macrophage-competent mice (SCID Fox Chase mice) injected with Raji cells and treated with rituximab.[82] However, it seems that HDAC6 inhibitor does not induce *MS4A1* transcription, but only increases *MS4A1* mRNA translation. Its potential clinical use is, therefore, likely limited to malignancies that have highly active *MS4A1* transcription but escape anti-CD20 antibodies by preventing its translation. This is not likely in most CLL cases, although, one report has suggested that *MS4A1* mRNA might be repressed post-transcriptionally by microRNAs in CLL.[83]

Regulation of CD20 levels on the cell-surface during therapy

Besides transcriptional and epigenetic regulation, several studies have demonstrated CD20 downmodulation on the B-cell surface in response to anti-CD20 therapy (Figure 1). One of these mechanisms is called "shaving". Monocytes and macrophages recognize rituximab binding to CD20 and remove this complex from the B-cell surface via the FcγRI-dependent process of endocytosis called trogocytosis[53] and this was observed in CLL patients treated with rituximab *in vivo* (Figure 1).[53,84,85] Alternatively, the acute change in CD20 levels in the malignant B-cell population after rituximab infusion might be partially due to elimination of those cells with the highest CD20 levels. Indeed, we have shown that after rituximab infusion *in vivo*, the antibody primarily targets and eliminates a subpopulation of CLL cells with the highest levels of CD20 via CDC, whereas many CLL cells with pre-therapy low CD20 levels survive.[34] Importantly, the CLL cells with the highest cell-surface CD20 levels are also those with the highest BCR signaling propensity and also represent the vast majority of Ki67-positive cells in peripheral blood.[34] This "targeting" of the most aggressive intraclonal CLL cell subpopulation at least partially explains the good clinical efficacy of rituximab. It remains to be determined whether rituximab is also targeting specific intraclonal cell subpopulations in diseases such as follicular lymphoma and DLBCL, in which malignant cells have relatively homogenously higher CD20 cell-surface levels.

Another mechanism reducing CD20 expression on B cells in response to anti-CD20 therapy is known as antigenic modulation (Figure 1).[86] This refers to the active internalization and subsequent degradation of CD20/monoclonal antibody complexes demanding energy and cytoskeleton remodeling. Importantly, only type I anti-CD20 monoclonal antibodies induce marked antigenic modulation. These anti-CD20 monoclonal antibodies (e.g. rituximab and ofatumumab) recognize and bind CD20 epitope in a different orientation than type II antibodies (obinutuzumab) and are able to redistribute CD20 into lipid rafts on the plasma membrane.[87,88] Type I anti-CD20 monoclonal antibodies also have an approximately two-fold higher capacity to bind CD20 epitope, which makes them prone to internalization and proteolytic degradation.[86,89] Moreover, the extent of antigenic modulation depends on the type of B-cell malignancy. The most rapid internalization can be seen in CLL cells, followed by mantle cell lymphoma cells, while follicular lymphoma and DLBCL cells show relatively lower rates of antigen internalization.[86] Lim *et al.* suggested that different rates of internalization in B-cell malignancies are due to different levels of inhibitory FcγRIIb on B cells (predominantly expressed on CLL and mantle cell lymphoma cells).[90] Rituximab was proposed to crosslink CD20 and FcγRIIb on the same B cell, resulting in FcγRIIb phosphorylation, and internalization of these complexes into lysosomes for their degradation.

The selection pressure caused by rituximab therapy can also lead to the emergence of malignant B-cell clones that are relatively or fully negative for cell-surface CD20 expression (Figure 1). In some DLBCL patients, mutations in the *MS4A1* coding sequence were identified; however, mutations involving rituximab epitope are extremely rare.[91] Terui *et al.* analyzed CD20 mutations in samples obtained from patients with previously untreated or relapsed/refractory B-NHL and found *MS4A1* mutations in 11 out of 50 patients (22%).[92] Importantly, in four cases (8%), such mutations resulted in a C-terminal truncated form of CD20 protein and reduced its cell-surface expression. Nakamaki *et al.* also reported a case of a relapsed DLBCL patient with a homozygous *MS4A1* gene deletion after rituximab-based therapy.[93]

Notably, some recurrent genetic mutations in patients with B-cell malignancies might affect CD20 levels, and be favored during therapy. In a clinical trial comparing fludarabine and cyclophosphamide treatment with fludarabine, cyclophosphamide and rituximab treatment, it was found that *NOTCH1* mutations are associated with a relative resistance to the anti-CD20 therapy.[94] Pozzo *et al.* showed that *NOTCH1*-mutated CLL cells are characterized by a lower CD20 expression in comparison to that of *NOTCH1*-wildtype CLL cells.[95] Mutations in NOTCH1 intracellular domain (NICD) result in dysregulation of HDAC-mediated epigenetic repression of CD20 through interactions with the RBPJ transcription factor. RBPJ acts as a negative regulator when forming a complex with HDAC1/2; however, accumulation of mutated NOTCH1 in the nucleus results in the preferential formation of NICD-RBPJ activating complex and higher HDAC1/2 levels available for interactions with an *MS4A1* promoter.

Recently, microenvironmental interactions in various B-cell malignancies were brought into focus as these provide essential pro-proliferative and pro-survival signals and promote drug resistance (reviewed by Seda & Mraz[48]). Interactions between mesenchymal stromal cells and CLL cells were shown to protect the leukemic cells from rituximab-induced CDC[96] and direct apoptosis,[97] and this can be therapeutically targeted by integrin inhibition (Figure 1).[97] These observations led to the coining of the term "cell adhesion-mediated antibody resistance" as an analogy to the long-known "cell adhesion-mediated drug resistance", which refers to resistance to classical chemotherapy. CD20 down-modulation in response to microenvironmental stimuli might be a theoretical explanation for cell adhesion-mediated antibody resistance. This is supported by the observation that stimulating normal B cells by co-culture with CD40L-expressing fibroblasts results in rapid CD20 endocytosis and thus reduces the cell-surface levels of CD20.[98] Additionally, Kawabata

et al. observed in the Ramos cell line that TGFβ signaling led to SMAD2/3 binding directly to the *MS4A1* transcription start site, resulting in CD20 repression.[99] However, we and others have shown that in CLL, the chemokine CXCL12 (also known as SDF1) produced by stromal cells in immune niches induces CD20 expression, and that the intraclonal CLL cell subpopulation that recently exited the lymph nodes is characterized by high levels of CD20.[34] This has an important consequence for the mechanism of rituximab's action since, *in vivo*, rituximab infusion leads to rapid and preferential elimination of this aggressive, proliferative CLL cell subpopulation. The remaining large proportion of CLL cells can survive the rituximab therapy because of relatively weak cell-surface levels of CD20, but these cells have a gene-expression profile of non-activated CLL cells, which are relatively less able to activate the BCR pathway, and do not proliferate. It remains unclear which molecular pathways provide CLL cells in the lymph node microenvironment with resistance to rituximab, despite having high levels of CD20.[97] The resistance to rituximab in the microenvironment seems to be limited to rituximab-mediated apoptosis and CDC.

Combinatorial therapy of novel drugs and anti-CD20 monoclonal antibodies

For over a decade, scientists and clinicians have become accustomed to the empirical experience that adding rituximab to other therapies leads to increased therapeutic efficacy in B-cell malignancies. This also prompted studies for strategies to induce higher CD20 levels on the B-cell surface to potentially sensitize malignant cells to anti-CD20 monoclonal antibodies (summarized in Table 2). Several of these "CD20 inducers" are being explored in preclinical or phase I/II clinical trials, including aurora kinase inhibitors, FOXO1 inhibitors, and chromatin modulators. For example, it has been shown that in lymphomas, the aurora kinase inhibitor alisertib can be safely and successfully used in combination with vincristine and rituximab (phase I/II trial).[72] Nevertheless, none of the "CD20 inducers" has been prioritized for phase III trials yet (see Table 2). Combining the BTK inhibitor ibrutinib with rituximab was expected to increase the BTK inhibitor's clinical efficacy. This hypothesis was supported by the observation that CLL/lymphoma cells become more sensitive to apoptosis and anti-CD20 monoclonal antibodies when mobilized from immune niches (a typical effect of BCR inhibitors).[96,97] However, results from a phase II study[12] demonstrated no benefit from adding rituximab to ibrutinib. Recent studies have shown that CD20 levels are repressed during ibrutinib therapy and that ibrutinib affects cells responsible for effector mechanisms such as T/NK cells and macrophages. The reduction of CD20 levels by ibrutinib has a clear impact on rituximab-mediated CDC and apoptosis. However, CD20 is not completely lost, which still allows for anti-CD20 monoclonal antibodies to bind to cells. Skarzynski *et al.*[100] also suggested that ibrutinib reduces complement inhibitor CD55 levels, which might partially counterbalance the effects of lower CD20, but it seems that in the sum of all effects, rituximab or ofatumumab efficacy decreases during ibrutinib therapy.

CD20 levels appear to play an essential role in CDC induced by rituximab, but they seem to be less relevant for ADCC.[101] Unfortunately, ibrutinib also affects the functions of T cells and NK cells by inhibiting their BTK or a related ITK. BTK is critical for regulating the functions of NK cells as BTK-less NK cells have impaired cytotoxic activity.[102] ITK signals downstream of the T-cell receptor and is required to activate NK cells through FcγRIII.[103] Inhibiting BTK or ITK impairs the cytotoxic functions of NK cells (degranulation, cytokine secretion) and ADCC mediated by type I and II anti-CD20 monoclonal antibodies.[104,105] Thus ibrutinib may impair T- and NK-cell functions through either BTK or ITK, or both.[104] It has also been suggested that phagocytosis by macrophages is affected by ibrutinib, but it is unclear if this is due to BTK inhibition in these cells or an off-target effect.[104-106] Based on the results from the iLLUMINATE study,[49] the Food and Drug Administration has already approved the combination of ibrutinib plus obinutuzumab for treatment-naïve patients with CLL (Table 2). However, the control arm of the study with chlorambucil plus obinutuzumab did not allow a conclusion on whether obinutuzumab provided a real benefit. Alternatively, sequential ibrutinib administration after the anti-CD20 monoclonal antibody could conceivably allow for better antibody effects. However, a clinical trial in CLL showed that sequential treatment with ofatumumab before ibrutinib was inferior to starting ibrutinib first, followed by the administration of ibrutinib and ofatumumab.[107] Since obinutuzumab acts in part through mechanisms different from those of type I antibodies (rituximab, ofatumumab), its combination with ibrutinib may lead to different effects and increased clinical efficacy. Alternatively, other BTK inhibitors, such as acalabrutinib, are more selective with less off-target activity and likely do not interfere with antibody-dependent cellular phagocytosis or ADCC.[108] It needs to be determined whether acalabrutinib is more suitable than ibrutinib for therapeutic combination with anti-CD20 monoclonal antibodies. Recently, a phase III study showed that acalabrutinib combined with obinutuzumab is highly efficient in prolonging progression-free survival when compared to obinutuzumab and chlorambucil in patients with previously untreated CLL.[109]

An interesting case is also the PI3Kδ inhibitor idelalisib, which is currently approved for use in combination with rituximab to treat relapsed CLL based on a comparison to rituximab alone (Table 2).[110] A similar phase III study showed that the progression-free survival of participants treated with a combination of idelalisib and ofatumumab was significantly longer than that of the group treated with ofatumumab only (Table 2).[111] However, it is unclear whether adding rituximab or ofatumumab to idelalisib actually provides any clinical benefit, and based on the understanding of CD20 regulation, it is very likely that PI3K inhibitors also repress CD20 expression. In fact, data indicate that any inhibitor repressing Akt or NFκB or CXCR4 activity in B cells, such as a SYK inhibitor, BTK inhibitor, PI3Kδ inhibitor, or CXCR4 antagonist, also reduces CD20 expression, leading to decreased binding of anti-CD20 monoclonal antibodies.[44,70,100,112,113] Notably, SRC inhibitors such as dasatinib also repress CD20 transcription and impair NK cell functions.[112] Additionally, PI3Kδ plays a critical role in maturation, development, and effector functions of NK cells, which would indicate that it might impair ADCC. However, some studies indicate that this might be less prominent than ibrutinib's effects[114] or that idelalisib does not reduce ADCC at all.[115]

Idelalisib was also shown to decrease IFNγ production by NK cells, and reduce secretion of various cytokines by T cells (IL6, IL10, TNFα, and CD40L).[115] More studies will be needed to determine whether other BTK or PI3K inhibitors have a better profile in terms of affecting ADCC/antibody-dependent cellular phagocytosis, but it seems inevitable that they will all lead to reduced levels of CD20.

The BH3-mimetic venetoclax was also tested in combination with anti-CD20 monoclonal antibodies. It seems that in contrast to "BCR inhibitors", there are no obvious biological reasons preventing an additive or synergistic effect of such a combination. The Food and Drug Administration granted approval for the use of venetoclax in combination with obinutuzumab for patients with previously untreated CLL/small lymphocytic lymphoma and in combination with rituximab to treat patients with CLL/ small lymphocytic lymphoma who have received at least one prior therapy (Table 2).[116] The biological rationale for such combinations in CLL is provided, among others, by a study showing that combining venetoclax and anti-CD20 monoclonal antibodies overcomes microenvironment-mediated resistance of CLL cells to venetoclax monotherapy *in vitro*.[117]

Conclusion

Although CD20 is considered to be an ideal therapeutic target and rituximab-based immunotherapy has become a standard of care for a majority of B-cell malignancies, it is still unclear what all the functions of CD20 are, and how its expression is regulated. The main reason is the large heterogeneity of patients with B-cell malignancies and the lack of mouse models with an evident phenotype which makes CD20 analysis *in vivo* more difficult. A full understanding of the complexity of regulation of CD20, its physiological function and the exact mechanism of action of anti-CD20 monoclonal antibodies is of pivotal importance to develop new modified anti-CD20 monoclonal antibodies and their therapeutic combination that would yield better clinical efficacy and/or less toxicity. Recently, the possible role of CD20 in microenvironmental interactions was underscored by the observation that CD20 is upregulated in the context of immune niches. This is likely to be of physiological importance, especially for BCR signaling; however, it is unclear if this is related to the putative function of CD20 as a regulator of calcium flux triggered by BCR or any potential role in T-cell interactions or some additional function(s). Further investigation of the physiological function of CD20 is required, including the identification of molecules that interact with CD20, since this has implications for the development of rational therapeutic combinations and strategies.

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