The Tumor Microenvironment Regulates CD19 and CD20 Immunotherapy for Lymphoma

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Abstract: B cells have diverse functions during immune responses, including antibody production, antigen presentation, and cytokine secretion. Multiple lymphomas and leukemias derive from malignant B cells, so therapies that deplete B cells are clinically important, particularly antibodies targeting the B cell–specific surface molecules CD19 and CD20. Macrophages are the principal mediators of CD19 and CD20 monoclonal antibody–dependent B-cell and lymphoma depletion in mice through Fcγ receptor–dependent phagocytosis. Thereby, the extent of CD19 or CD20 antibody–induced B cell and tumor depletion in vivo is influenced by molecular changes within tumors and genetic variations between individuals. In addition to Fcγ receptor polymorphisms, lymphoma- and regulatory B cell–derived cytokine production and macrophage localization and function within tumor microenvironments influence tumor clearance. Given the dynamic interactions of these factors, the identification of effector cell and tumor microenvironment genetic alterations will identify molecular targets that enhance immunotherapies for the treatment of human diseases.

Key Words: Antibody, antibody-dependent phagocytosis, B10 cell, B cell, CD19, CD20, FcγR, immunotherapy, lymphoma, monoclonal antibody


The immune system predominantly serves to protect the body from microorganisms and cancer development. However, like other dividing cells in the body, immune cells can also become malignant and develop into cancer and may also become ineffective or even tumor promoting within the tumor microenvironment in the presence of anti-inflammatory mediators. Non-Hodgkin lymphoma (NHL), which is the most common hematologic malignancy and accounts for approximately 4% of all adult cancers in the United States, mostly derives from B cells.2,3 Whereas B cell NHLs vary by the transformative events leading to malignancy and growth patterns, most express cell surface antibody (Ab) antigen receptors, CD19, and CD20.2 More than 90% of human B cell lymphomas and most B-cell leukemias express CD19 and CD20,2 making these molecules ideal targets for malignant B cell immunotherapy.5 In addition, autoimmunity and other conditions that are worsened by B cell effector functions, excessive or inappropriate Ab production, or B cell–derived cytokines have made therapeutic B cell depletion an attractive treatment option for multiple clinical scenarios. CD19 and CD20 are members of a small subset of B cell–restricted cell surface glycoproteins. The specificity of CD19 and CD20 expression among B cells alone makes them ideal candidates for Ab immunotherapy targets to specifically deplete endogenous and malignant B cells in vivo. Indeed, the most common therapies currently used to treat NHL include CD20 monoclonal Abs (mAbs). Rituximab and other chimeric and radioiodinated CD20 mAbs4,9 and CD1910–15 mAbs are available to treat NHLs and deplete endogenous B cells in rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, hemolytic anemia, and other autoimmune conditions.16–20 However, it is now appreciated that the initial effectiveness of mAb therapy varies widely among patients and typically wanes over time, despite sustained target molecule expression by malignant cells among the vast majority of relapsing patients.21–23 Although mAb-based therapies are now widely used, the precise mechanisms regulating effective mAb-based lymphoma clearance are not well understood. This review highlights some of the known molecular events that regulate mAb-dependent lymphoma depletion within the tumor microenvironment in order to provide guidance for future studies.

CD19 and CD20 Expression and Functions During B cell Development

B cells first express CD19 during pre–B cell development, which then increases throughout B cell maturation, most notably from the transition from immature to mature B cells, until plasma cell differentiation.24–26 Human and mouse CD19 expression patterns are similar, except mouse CD19 is expressed at high levels by mouse pre–B cells, whereas human CD19 expression densities increase gradually starting at the late pro–B cell stage throughout pre–B cell development in both humans and human CD19 transgenic mice.27–30 Both human and mouse CD19 expression persist during the early differentiation of mature B cells into plasma cells, with the eventual loss of most CD19 expression by terminally differentiated plasma cells.10 CD20 expression begins later than does CD19, during the transition from pre–B cell to immature B cell and continues through maturation until very early plasmablast differentiation in both humans31–34 and mice.35,36 Thus, even though CD19 and B220/CD45R are used as common B cell markers in mice, whereas CD19 and CD20 are used as human B cell markers, the anti–mouse CD19 and CD20 immunotherapy data generated in mice readily translate into the human setting, given the common patterns of CD19 or CD20 expression between species.36

CD19 is a costimulatory molecule that regulates B cell signaling thresholds during negative selection and clonal expansion as well as B cell development.37–40 CD19 also critically regulates early lymphomagenesis by stabilizing the expression of the proto-oncogene c-myc.41,42 CD20 plays a role in B cell activation and proliferation by regulating transmembrane calcium conductance and subsequent cell cycle progression following activation.43,44 CD20, however, is not explicitly required for B cell development or tissue localization,45,46 whereas CD19 is a critical regulator of signaling thresholds during B cell development and function.47,48,49

CD19 and CD20 Immunotherapies

The significance of CD19 and CD20 in B cell function is reflected in the magnitude and longevity of B cell depletion by
mAbs targeting these molecules. In mice, a single dose of either CD19 mAb (immunoglobulin G2a [IgG2a]) or CD20 mAb (IgG2c) depletes more than 90% of B cells from the circulation and lymphoid tissues within 1 week of mAb treatment.\textsuperscript{10,26,48,49} Because of pre-B cell depletion by CD19 mAbs, B cell numbers begin to recover much more rapidly in mice depleted of B cells using CD20 mAb, which occurs 7 to 8 weeks after mAb treatment, than in mice treated with CD19 mAb, which occurs 11 to 15 weeks after therapy.\textsuperscript{10,26,49} At the other end of B cell maturation, CD19 mAb depletes both short-lived plasmablasts and some long-lived plasma cells and thereby impairs primary and secondary Ab responses to T cell–independent and –dependent antigens and autoantibody development while reducing circulating IgM, IgG, and IgA levels during homeostasis in human CD19 transgenic mice.\textsuperscript{40,50} By contrast, B cell depletion by CD20 mAb does not affect serum Ab levels or bone marrow pre-B cells or plasma cell numbers in wild-type mice, in accordance with the known expression pattern of CD20.\textsuperscript{35,49,51} CD19 and CD20 mAbs also deplete the vast majority of mature B cells,\textsuperscript{10,55} including memory B cells, although approximately 10% and 30% of germinal center B cells remain following mAb treatment.\textsuperscript{35,52} Both CD19 and CD20 mAbs also deplete malignant B cells, in addition to depleting endogenous B cells.\textsuperscript{53,54}

It has been difficult to determine the efficacy of B cell depletion in humans as most studies focus on circulating blood B cells, which account for less than 2% of total B cells\textsuperscript{55} and may therefore neglect the impact of B cells in various lymphoid tissues. Furthermore, as many patients see little therapeutic benefit from mAb treatment and many more individuals become unresponsive to treatment,\textsuperscript{21–23} consideration of differences in B cell depletion across different tissues and the likely existence of malignant B cell reservoirs becomes critically important. Given this caveat and the immense genetic diversity in human patients, attempts to elucidate the mechanism through which B cells are depleted in humans have generated varied results. In select patients, decreased CD20 expression density may account for ineffective CD20 mAb therapy of malignant B cells.\textsuperscript{56} Otherwise, binding of both CD19 and CD20 mAbs has been suggested to alter cell cycle progression and induce apoptosis.\textsuperscript{44,57–59} It has also been suggested that CD19 and CD20 mAb–based depletion of human B cells and lymphomas occurs through innate immune system activation by inducing complement- or Ab-dependent cytolysis.\textsuperscript{14,15,59–66} Some studies have shown that CD20 mAb treatment of recently isolated lymphoma cells or immortalized B cell lines drives classic pathway complement activation and complement-dependent cytotoxicity.\textsuperscript{52,67–69} Furthermore, CD20 mAb activates complement in some patients.\textsuperscript{70} However, other studies have found that neither the observed complement-mediated lysis nor lymphoma expression of complement inhibitors (e.g., CD46, CD55, CD59) predicts therapeutic outcome.\textsuperscript{71} Indeed, studies in nude mice have shown that while complement depletion has no effect on mAb treatment of human melanoma or carcinoma, inhibition of macrophage effector function eliminates mAb-dependent tumor depletion.\textsuperscript{72} Furthermore, although human natural killer (NK) cells are frequently used as effector cells in vitro during mAb-dependent tumor lysis assays for therapeutic mAbs, there remains no direct evidence that NK cells mediate mAb-dependent tumor killing in vivo.\textsuperscript{36}

**Macrophages Mediate mAb-Dependent Mouse B Cell Depletion In Vivo**

In mice, CD19 and CD20 mAbs predominantly, if not exclusively, deplete B cells through FcγR receptor (FcγR)–dependent monocyte function. Mice with reduced tissue macrophage numbers following liposome-encapsulated clodronate treatment, which predominantly depletes macrophages in the spleen and liver, clear far fewer endogenous and malignant B cells than wild-type mice following CD19 or CD20 mAb treatment.\textsuperscript{10,48,53,54} Kupffer macrophages in the liver have also been directly observed to clear endogenous and malignant B cells following mAb therapy through
Ab-dependent phagocytosis.\textsuperscript{73,74} The mAb-dependent depletion of endogenous and malignant B cells by macrophages can be augmented by activating macrophage pattern recognition Toll-like receptors (TLRs) with poly(I:C) (TLR3/TRIF agonist), lipopolysaccharide (TLR4 agonist), and CpG (TLR9 agonist) in vitro.\textsuperscript{54} However, stimulation with poly(I:C) is preferential to other TLR agonists in vivo, because poly(I:C) specifically enhances macrophage phagocytosis and cytotoxic molecule expression, including tumor necrosis factor $\alpha$ and nitric oxide (Fig. 1). While complement activation has been suggested as a mechanism by which human B cells are depleted following CD20 mAb therapy, mice deficient in C1q, C3, or C4 clear B cells following CD20 mAb treatment at rates similar to wild-type mice.\textsuperscript{46,51,54} Furthermore, deficiencies in T cells, peritoneal, neutrophils, or functional NK cells do not impact CD20 mAb–dependent depletion of endogenous or malignant B cells. Thus, macrophages deplete endogenous and malignant B cells following CD20 mAb therapy predominantly through Ab-dependent mechanisms (Fig. 1). It is difficult to distinguish between Ab-dependent cell phagocytosis and other cytotoxic mechanisms for B cell depletion in vivo because it remains possible that Ab-induced cytotoxic agents may actually kill the B cells prior to Ab-dependent phagocytosis. Regardless, macrophages are the critical mediators of either process.

**FcγR Expression Is Critical for Lymphoma Depletion**

The density and type of FcγR expressed by macrophages are critical to the outcome of CD19 and CD20 mAb treatment, as mice deficient in FcγRs do not deplete B cells following CD19 or CD20 mAb therapy.\textsuperscript{10,48,51,54} FcγRI, FcγRII, and FcγRIIb cooperate to mediate mAb-dependent B cell depletion and can compensate for deficiencies in any of these receptors,\textsuperscript{10,48,51,53,54} whereas the inhibitory FcγRIIB hinders mAb-dependent B cell depletion.\textsuperscript{51,53} Furthermore, polymorphisms in FcγR among different mouse strains alter the efficacy of mAb-based B cell depletion. NOD mice, which have a truncated FcγRI and polymorphisms in FcγRII and FcγRIV relative to C57BL/6 mice, deplete fewer B cells following CD20 mAb treatment.\textsuperscript{75} Thus, macrophage expression of diverse FcγRs is critical for optimal B cell and lymphoma depletion by CD19 and CD20 mAbs (Fig. 1).

While diverse FcγRs are required for optimal CD19 and CD20 immunotherapy outcomes, the efficacy of the different isotypes of CD19 and CD20 mAbs is distinct. CD19 mAbs of the IgG1, IgG2a, and IgG2b isotypes deplete B cells similarly, likely owing to the higher density of CD19 expression on the surface of most B cells; however, IgA CD19 mAbs do not deplete B cells in vivo.\textsuperscript{10} By contrast, the outcome of CD20 mAb–dependent B-cell depletion does correlate with mAb isotype, which occurs as follows: IgG2a/c $>$ IgG1 $>$ IgG2b $>$ IgG3, although IgG3 CD20 mAbs do not deplete B cells effectively.\textsuperscript{56,58} Thus, IgG isotype becomes more important as mAb concentrations are reduced and/or target molecule densities are lower. However, the combination of low doses of both CD19 and CD20 mAbs depletes more B cells in the spleen and lymph nodes than either mAb at a comparable dose accomplishes alone,\textsuperscript{10} indicating that combination therapies using both mAbs have emergent effects that may prove important for clinical treatment regimens.

In humans, polymorphisms in the activating FcγRIIa and FcγRIII proteins have been correlated with the efficiency of B cell and tumor depletion during CD20 mAb therapy in some lymphoma patients.\textsuperscript{76–78} FcγRIIa and FcγRIII are both expressed by human cells of the myeloid lineage, including macrophages, neutrophils, dendritic cells, and NK cells, perhaps indicating that some or all of these cell populations contribute to mAb-dependent cell depletion.\textsuperscript{80} Deletion of inhibitory FcγRIIB exacerbates cell-mediated cytotoxicity of humanized mAbs toward human lymphoma cells in nude mice in vivo,\textsuperscript{63} although no relationship has been found between FcγRIIB protein density and patient prognosis.\textsuperscript{79} Regardless, other factors are likely to also contribute to therapy variations between humans.

**Protected Microenvironments for B Cells and Lymphoma Cells**

CD19 and CD20 mAbs rapidly deplete the vast majority of circulating B cells in mice, with subsequent B cell depletion within lymphoid tissues, although some residual B cells are observed depending on the effectiveness of the specific mAb that is used and the mAb dose.\textsuperscript{10,35,49,51} The residual B cells found within most lymphoid tissues 1 week after potent CD19 or CD20 mAb treatments range between 1% and 5% of B cells and are pro–B cells, pre–B cells, and phenotypically immature B cells typically found in the bone marrow.\textsuperscript{10,35,49,51} Some plasmablasts are also detectable within tissues following mAb treatment as CD20 is quickly down-regulated during terminal plasma cell differentiation, with expression of CD19 persisting longer than CD20.\textsuperscript{10,35,49,51}

Peritoneal cavity B cell depletion by CD19 and CD20 mAbs is much less effective than in other lymphoid tissues despite high CD19 and CD20 expression densities and high levels of mAb binding by peritoneal B cells.\textsuperscript{28,49} Peritoneal cavity B cells are reduced by only 60–70% and 30% following immunotherapy with potent CD19 and CD20 mAbs, respectively, with B1a cells showing a particular resilience to depletion.\textsuperscript{28,49} Delayed peritoneal cavity B cell depletion by CD19 and CD20 mAbs suggests that mAb binding to these target molecules alone does not induce B cell–intrinsic changes or apoptosis. Instead, inefficient B cell depletion by CD19 and CD20 mAbs reflects the absence of effector cells in the peritoneal cavity, as the defect in B cell clearance can be largely reversed by the recruitment of inflammatory macrophages into the peritoneum following thioglycolate injection, although peritoneal B2 cells are still more rapidly cleared than B1 cells.\textsuperscript{49} Differential B cell depletion within specific microenvironments may also have important consequences for mAb treatment of human disease, as malignant B cells within tissues such as the peritoneal cavity may not be depleted as quickly as circulating B cells and could serve as an important reservoir for recrudescent tumors.

**Immunosuppressive Cytokine Production Within Tumor Microenvironments**

Primary and recrudescence tumors can actively suppress ongoing antitumor immune responses within tumor microenvironments through multiple mechanisms, including the expression of immunosuppressive cytokines. As an example, patients with chronic lymphocytic leukemia (CLL) are less responsive to CD20 immunotherapy than are those with many other NHLs. However, within the vast majority of patients with CLL, some CLL cells are competent to secrete the negative regulatory cytokine interleukin 10 (IL-10) following appropriate stimulation.\textsuperscript{60} The plasma IL-10 levels of patients with CLL are also frequently elevated relative to those of control subjects, although plasma IL-10 levels do not correlate with numbers of IL-10–competent CLL cells, suggesting that other cells may also produce IL-10. Thereby, unknown triggers may induce CLL cell IL-10 production in vivo, which might promote immune privilege through the inhibition of monocytic-mediated lymphoma cytotoxicity or phagocytosis (Fig. 1).

CLL cells resemble a subset of naturally occurring regulatory B cells called B10 cells that are competent to produce IL-10.\textsuperscript{80,81} There is a dramatic age-associated expansion of regulatory B10 cells in the transgenic TCL1 (T-cell leukemia protein 1) mouse
model of CLL, where mice frequently develop CLL-like disease by 1 year of age. Remarkably, the cell surface phenotype of the expanded B10 cell subset in aged TCL1 mice parallels the phenotype of CLL cells, suggesting a shared functional program and/or overlapping developmental relationship between these cells. These IL-10–competent CLL-like cells inhibit macrophage cytokine production in an IL-10–dependent manner and produce IL-10 in response to low-dose inflammation, suggesting that IL-10 expression by lymphoma cells significantly impairs the antitumor immune response (Fig. 1).

Endogenous B10 cells also profoundly impede the efficacy of CD20 mAb therapy and inhibit B cell lymphoma depletion. Thereby, reducing B10 cell numbers significantly contributes to the effectiveness of CD20 mAb treatment for lymphoma therapy. In cases of incomplete tissue B-cell depletion, as likely occurs in most patients receiving CD20 immunotherapy, the inadequate clearance of B10 cells reduces the effectiveness of mAb-based treatment, which can have profound functional consequences for mAb therapy in mouse models. In particular, B10 cell–derived IL-10 ablates macrophage activation and cytokine production (Fig. 1). However, enhancing macrophage function by treating mice with the TLR9 agonist poly(I: C) is able to significantly enhance lymphoma cell clearance. Poly(I: C) is effective in this capacity as it uniquely augments macrophage but not B10 cell activation, whereas other TLR agonists such as lipopolysaccharide or CpG oligonucleotides activate B10 cells, which is counterproductive. Thus, IL-10 derived from endogenous B10 cells and potentially malignant cells can suppress CD19 and CD20 immunotherapy. Other cytokines and regulatory molecules produced within the tumor microenvironment are likely to have similar negative effects on lymphoma clearance.

CONCLUSIONS

As both CD19 and CD20 mAbs deplete B cells through mAb-dependent effector mechanisms that require macrophages, treatments that augment macrophage numbers, function, and phagocytosis offer potential means to improve the efficacy of these and other immunotherapies. As an example, regulatory B10 cells impair CD20 immunotherapy of lymphoma by inhibiting macrophage activation. Thereby, immunotherapeutic B10 cell depletion would likely enhance mAb-dependent lymphoma clearance as occurs with poly(I: C) treatment. Given the emerging importance of T cell checkpoint inhibitors in cancer treatment, it remains possible that these immunotherapies may also improve outcomes in lymphoma patients given CD19 or CD20 mAbs. Similarly, the development of checkpoint inhibitors that preferentially augment macrophage activation and function may dramatically enhance lymphoma and solid tumor clearance by the growing array of FcyR-dependent therapies.

Because the initial effectiveness of CD19 and CD20 immunotherapies varies widely among patients and typically wanes over time regardless of target molecule expression, it will be imperative to identify the spectrum of genetic factors that drive macrophage effector mechanisms and the promotion of an effective antitumor microenvironment within patients. The comprehensive interrogation of the tumor-intrinsic genetic network that suppresses antitumor immune responses will also yield valuable therapeutic targets for augmenting CD19- and CD20-focused therapies. Undoubtedly, many of the genetic changes within tumors and genetic differences between patients that influence CD19 and CD20 immunotherapies will also dictate the effectiveness of other treatments such as chimeric antigen receptor–bearing T cells. Thereby, a better genetic and molecular understanding of the interactions between immune cells and lymphomas following CD19 and CD20 immunotherapy within the tumor microenvironment will undoubtedly improve the efficacy of current immunotherapies and may lead to the discovery of new strategies for depleting lymphomas and other malignant cells within tumor microenvironments.

REFERENCES


