Am"B"valent: anti-CD20 antibodies unravel the dual role of B cells in immunopathogenesis

Running title: Dual role of B cell in immunopathogenesis

Olivier Thaunat\textsuperscript{1, 2, 3, 4}, Emmanuel Morelon\textsuperscript{1, 2, 3, 4} & Thierry Defrance\textsuperscript{1, 3, 4}

1) Université de Lyon, Lyon 1, Villeurbanne, France
2) Hospices Civils de Lyon, Hôpital Edouard Herriot, Service de Transplantation Rénale et d'Immunologie Clinique, F-69437 Lyon, France
3) INSERM U851, 21 Avenue Tony Garnier, Lyon, F-69007, France
4) IFR128, Biosciences Lyon-Gerland, Lyon, F-69007, France

Corresponding author:
Olivier Thaunat
Service de Transplantation Rénale et d'Immunologie Clinique
Hôpital Edouard Herriot
5 place d'Arsonval
69437 Lyon Cedex 03
Tel: +33(0) 1 55 42 82 69
Fax: +33(0) 1 55 42 82 62
Email : olivier.thaunatpastu@free.fr

Copyright © 2010 American Society of Hematology
Summary

Accumulating evidence has designated B cells as central players in the pathogenesis of immune diseases. In the late 1990s, anti-CD20 monoclonal antibodies were developed for the treatment of B-cell non-Hodgkin’s lymphomas, offering the opportunity to efficiently deplete the B-cell compartment for therapeutic immunointerventions. Several studies have since established the beneficial effect of this drug on the course of a wide range of immune diseases. However, paradoxically, it has also been reported that rituximab sometimes worsens the symptoms of the very same conditions.

The explanation that reconciles such apparently conflicting results has recently emerged from basic studies, which demonstrate i) that B cells are also endowed with immune regulatory properties and ii) that the opposing contributions of B cells may overlap during the course of the disease.

Caution should therefore be exercised when considering B cell depletion since the therapeutic effect will depend on the relative contributions of the opposing B cell activities at the time of the drug administration.

Key words:

B cell, anti-CD20, rituximab, immune regulation
“Bad boys” B cells

Historically the role of B lymphocytes in the pathogenesis of immune diseases has been mainly associated with their capacity to produce harmful antibodies after differentiation into plasma cells. This conception was based on seminal experiments which demonstrated that the mere transfer of antibodies was sufficient to recapitulate the symptoms of myasthenia gravis, Graves’ disease, Goodpasture’s disease...etc ¹. In contrast to these diseases, other autoimmune conditions, in which the role of antibodies has not been recognized, have traditionally been termed “T cell-mediated” diseases. Among the latter, type 1 diabetes, in which T lymphocytes are crucial for the destruction of the β islets, has long been considered as archetypal. However, recent investigations in NOD mice have shown that more than 50% ² of the lymphocytes infiltrating islets of Langerhans are B cells ³ and that these B cells are critically necessary for the development of diabetes ⁴. Another clue that B cells exert pathogenic roles through “antibody-independent” mechanistic pathways came from genetically modified lupus-prone mice. While B cell depletion leads to abrogation of the disease in this model, transgenic mice, whose B cells cannot secrete immunoglobulin, still developed nephritis ⁵. Thus, in many immune diseases, even including those not driven by antibodies, B cells have been demonstrated to play an essential pathogenic role.

Among the “antibody-independent” pathogenic roles of B cells, accumulating evidence points to their capacity to present antigen ⁶. Upon recognition of specific antigen, the B cell membrane is reorganized resulting in the aggregation of BCR in an immunological synapse that functions as a platform
for internalization of the complex. Internalized antigen is degraded and subsequently exposed on the B cell surface in association with MHC molecules for presentation to T cells. This surface presentation of antigen, in the presence of various costimulatory molecules, elicits the T cell assistance required for B cell maturation, which in turn allows B cells to drive optimal T cell activation and differentiation into memory subsets (Figure 1, left panel).

Of note, B cells are endowed with unique properties as antigen presenting cells inasmuch as they have an antigen-specific receptor, allowing extraction and presentation of antigen, even if it is membrane-tethered or present in limiting quantities. Furthermore, B cells also have the capacity to clonally expand thereby becoming the numerically dominant antigen presenting cells. In addition to antigen presentation, activated B cells also produce a wide range of cytokines and chemokines that modulate the maturation, the migration and the function of others immune effectors. In particular, B cells have been shown to play a critical role in lymphoid neogenesis, i.e. the process by which ectopic functional lymphoid structures (“tertiary lymphoid tissues”) appear de novo during chronic inflammation. Several studies have demonstrated that tertiary lymphoid tissues are permissive microenvironments for the induction of immune responses and have led to the hypothesis that lymphoid neogenesis may contribute to the exacerbation of a wide range of chronic inflammatory diseases.

**Anti-CD20 monoclonal antibody for therapeutic B cell depletion**

Given the multiple pathogenic roles attributed to B lymphocytes, therapeutic strategies that aim at depleting this cell population were expected to be beneficial in a wide range of immune diseases.
Pioneer attempts to deplete B cells in the 1980s relied on xenogenic polyclonal antibodies directed against the surface immunoglobulin receptor. Finally, it was only in the late 1990s that progress achieved in the treatment of lymphoproliferative disorders offered clinicians the opportunity to efficiently target the B-cell compartment for therapeutic immunointerventions.

CD20 is a transmembrane protein that functions as a Ca-permeable cation channel, whose expression is restricted to B cells from the pre-B-cell to the immunoblast stage. Rituximab, a chimeric monoclonal antibody composed of human IgG1 kappa antibody with variable regions isolated from a murine anti-CD20 clone (IDEC-2B8) was initially introduced for the treatment of B-cell non-Hodgkin’s lymphomas. A single course of rituximab successfully depleted peripheral human B lymphocytes for periods ranging from three months to more than one year through mechanisms involving Fc- and complement-dependent killing as well as other signals inducing apoptosis (Figure 2, upper panel). Several studies have highlighted the influence of circulatory dynamics and microenvironment on the efficiency of rituximab depletion. As a result, the reduction of B cell numbers after rituximab appears to be less complete in secondary lymphoid tissues than in peripheral blood and variations in the extent and kinetics of the depletion have been reported among B cell subsets.

**Successes of therapeutic B cell depletion**

The most frequent haematological malignancy is non-Hodgkin’s lymphoma, 85% of which are of B-cell origin. Rituximab has been approved for the treatment of B-cell non-Hodgkin’s lymphoma in 1997. Since then, a number of randomized, phase III trials have reported significant survival benefits
associated with rituximab, in combination with chemotherapy, in patients with diffuse large B-cell lymphoma and follicular lymphoma. Furthermore, these benefits have been demonstrated for rituximab in combination with a wide variety of chemotherapy regimens, across many patient subtypes, and in different treatment and disease settings (for a recent review please see 27), leading to the conclusion that anti-CD20 monoclonal antibodies represent one of the most important advance in the treatment of B-cell lymphoma in the past 30 years.

In chronic lymphocytic leukaemia, the leukemic counterpart of small lymphocytic lymphoma, the addition of rituximab to fludarabine-based chemotherapy has significantly increased complete response and progression-free survival rates for both untreated and relapsed or refractory chronic lymphocytic leukaemia 27.

The association between autoimmune diseases and haematological malignancies has long been recognised. While autoimmune conditions associated with haematological malignancies can affect any organ, they seem to predominantly target blood constituents 28. The pathophysiology of these autoimmune manifestations is complex and remains incompletely understood. Autoimmune anemia, for example, can result either from monoclonal antibodies produced by the lymphoma clone (i.e. cold hemagglutinin disease), or can be the consequence of polyclonal autoantibodies produced by the residual non-malignant B cells in chronic lymphocytic leukaemia. Several reports suggest that treatments, in particular chlorambucil and fludarabine, might trigger the onset of autoimmune manifestations 29,30. In contrast, rituximab has been shown to be an effective treatment for chronic lymphocytic
leukaemia-associated autoimmune hemolytic anemia \(^{28,31}\), and for mixed cryoglobulinemia and cold agglutinins secondary to non-Hodgkin's lymphoma \(^{32}\).

These observations along with the favourable safety profile of the rituximab in patients treated for haematological malignancies \(^{33}\) has catalyzed the application of the drug in primary immune diseases. Several clinical studies have since established the beneficial effect of B cell depletion on the course of a wide range of immune diseases.

Rheumatoid arthritis, a multisystem disorder that predominantly causes inflammation in synovial joints, is the autoimmune condition in which the effect of rituximab has been the most studied \(^{34}\). In the pivotal phase IIa study, about 80% of patients with active rheumatoid arthritis (despite methotrexate treatment) showed clinical benefit after rituximab administration - a percentage similar to the one obtained with anti-TNF agents \(^{35}\). Furthermore, a recent phase III trial has reported that even patients with an inadequate response to anti-TNF agents showed significant improvement after rituximab therapy \(^{36}\), a result that prompted the FDA to approve the drug for the treatment of refractory rheumatoid arthritis.

In chronic idiopathic thrombocytopenic purpura, an acquired haemorrhagic condition associated with accelerated platelet consumption due to anti-platelet autoantibodies, a phase II study has reported 40% of good responses to rituximab \(^{37}\).

Based on previous encouraging case reports, a phase II controlled clinical trial has recently been conducted in multiple sclerosis. In patients with relapsing-
remitting multiple sclerosis, a single course of rituximab reduced inflammatory brain lesions and clinical relapses for 48 weeks. 38. Numerous small open studies and isolated cases have also reported favourable outcomes following rituximab administration in a wide range of autoimmune diseases including, vasculitis 39, autoantibody-associated neuropathies 40, Graves’ disease 41, myasthenia gravis 42, myositis 43, blistering skin disorders 44,45, mixed cryoglobulinemia 46, thrombotic thrombocytopenic purpura 47, Sjögren’s syndrome 48, and anti-factor VIII syndrome 49. There are ongoing phase II and III trials of rituximab for each of these autoimmune disorders 50.

Finally, recent advances in solid organ transplantation have unravelled new mechanisms of allograft damage. Unexpected clusters of CD20+ B cells have been discovered in rejected grafts 18,51 and C4d deposition, indicating classic complement pathway activation, is now routinely seen in refractory rejection 52. Rituximab has therefore emerged as a rational choice for therapy in transplantation to abrogate B-cell mediated events 53,54.

Limits of therapeutic B cell depletion

Given the central role of B cells, autoantibodies and immune complexes in the pathophysiology of systemic lupus erythematosus (SLE), it was widely anticipated that anti-CD20 would be efficient in treating SLE. However, despite the successes reported in some open-label preliminary studies 55,56, rituximab failed to meet its primary and secondary end points in two large controlled trials of non-renal SLE 57, and renal lupus nephritis 58. These disappointing results are sometimes attributed to the fact that the highly heterogeneous clinical presentation of SLE makes it difficult to devise
quantitative measures of response to therapy. However, the latter justification is an unlikely explanation for either the paradoxical exacerbation of the clinical condition \(^{59-61}\), or the onset of new immune diseases (including psoriasis \(^{62,63}\), vasculitis \(^{64-66}\), interstitial pneumonitis \(^{67-69}\), and auto-immune cytopenia \(^{70,71}\)) that are sometimes observed after rituximab administration. One could argue that these small non-randomized studies focus on a very rare adverse effect of rituximab, since this problem was not identified in the huge cohort of patients treated for hematological malignancies \(^{33}\). We rather favour the alternative explanation that rituximab-induced autoimmune complications are under-reported. It is indeed a well-established fact that the quality and quantity of drug safety reporting are inadequate \(^{72}\), a matter that seems even more common for drugs with excellent efficacy outcomes \(^{72}\). One can indeed conceive the reluctance of clinicians to report side effects of a drug that has changed the standard of care for patients suffering from life-threatening diseases such as non-Hodgkin’s lymphoma or chronic lymphocytic leukaemia. Furthermore, the widely recognised association between autoimmune diseases and haematological malignancies (already discussed above) makes very difficult the formal incrimination of the drug for these patients. The same concern also exists for patients receiving rituximab for an autoimmune disease, the occurrence of distinct autoimmune conditions in the same patients being a very common feature \(^{73}\). Thus, the current lack of consistent report demonstrating the increased incidence of autoimmune phenomena after rituximab administration does not exclude the possibility that a carefully conducted meta-analysis might still be able to detect this adverse event.
In this context, the results of the study recently published by Clatworthy et al are particularly interesting, inasmuch as they clearly establish the "paradoxical" immune stimulatory effect of rituximab. Indeed, this randomized controlled trial, which compared rituximab with an anti-CD25 monoclonal antibody as induction therapy in patients undergoing renal transplantation, had to be suspended because the authors observed a 6-fold increased incidence of acute cellular rejection in the rituximab group (83% versus 14%).

Based on the worsening of autoimmunity observed in chronic lymphocytic leukaemia patients receiving fludarabine, a drug that induces a profound lymphopenia, it is tempting to speculate that this "paradoxical immune stimulation" is rather a general feature following B cell depletion than a specific adverse effect of rituximab.

Finally, the ambivalency of B cell depletion on the course of immune diseases has been recently demonstrated in experimental autoimmune encephalomyelitis. Experimental autoimmune encephalomyelitis (EAE) is an autoimmune central nervous system disease that can be induced in certain susceptible murine strains following immunization with myelin to model human multiple sclerosis. While CD20 antibody–mediated B cell depletion during EAE disease progression dramatically reduced the symptoms; the same treatment given before EAE induction substantially exacerbated the disease (Figure 3). Interestingly, administration of the drug at other time points had no significant effect (Figure 3).

Altogether, these data demonstrate that it is more difficult than anticipated to forecast the effect of B cell depletion on the course of an immune disease: the
same therapy can lead to opposite outcomes depending on the timing of its administration

**Evidence for an immune regulatory role of B cells**

The explanation that reconciles such apparently conflicting results has recently emerged from basic studies that demonstrate an immune regulatory role of B cells.

The first clue that B cells can regulate immune responses was provided by seminal *in vivo* experimental studies by Shimamura et al, demonstrating that adoptive transfer of antigen-activated B cells could induce tolerance in naive mice through the induction of suppressor T cells. Despite these data, the role of B cells in the regulation of immune diseases remained overlooked for another decade, until Wolf et al reported that mice lacking B cells suffered an unusually severe and chronic form of EAE. While spontaneous recovery is the norm in wild-type mice, the authors observed that genetically B-cell-deficient mice of the same background experienced a greater variation in disease onset and severity, and failed to recover completely. Following this seminal contribution, independent groups made consistent observations in other experimental models of autoimmune diseases, including collagen-induced arthritis and a model of spontaneous colitis, pathologically reminiscent of human ulcerative colitis. Interestingly, while the pathogenic T-cell response involves the same T helper 1 (Th1) cells and Th17 pro-inflammatory T-cell populations in EAE and collagen-induced arthritis, the colitis model differs in that the inflammation appears to be driven by Th2 cells. Thus the B-cell compartment has the capacity to control organ-specific inflammation that may be driven by Th1-, Th2- or Th17-effectors.
Dissection of the underlying mechanisms revealed that B cells limit immune disease progression by providing interleukin 10 (IL10) \(^{75,78,80-82}\) that, in turn, directly suppresses the differentiation of pathogenic T-cells, promotes the development of regulatory T cells \(^{83}\) and constrains dendritic cell functions \(^{84}\) (Figure 1, right panel).

The recent demonstration that IL10-producing B cells exist in humans, and that B cells from patients with multiple sclerosis \(^{85}\) and lupus \(^{86}\) produce decreased amounts of IL10 suggests a general role of B cells in clinical immune homeostasis.

**Which B-cells control the immune responses?**

B cells can be stimulated to produce IL10 by a combination of ligation of the B-cell receptor by the antigen and of CD40 by CD40 ligand. Some reports also point to a critical non-redundant role of certain toll-like receptors (TLR2 & TLR4) in driving the regulatory activity in B cells \(^{87}\).

The involvement of the BCR, CD40 and TLRs in the regulatory function of B cells raises a conceptual difficulty. Indeed, these signals are the very same as the ones involved in the activation of B cells in most immune responses. One hypothesis would therefore be the existence of a peculiar "B reg" subset, endowed with the unique function to regulate immune processes. In the mouse, B cells are classically divided into B-1 cells that reside in pleural and peritoneal cavities and B-2 cells that populate secondary lymphoid organs. Peritoneal B-1 cells, known to produce particularly large amounts of IL10 following stimulation, have been ascribed with regulatory function in some studies \(^{88,89}\). However, since this subset was excluded from the transfer
experiments demonstrating the regulatory role of B cells \(^{78,80,90}\), it is likely that B-2 cells also contain a subset regulating immune diseases. Interestingly, a rare population of splenic B cells characterized by a unique phenotype that associates features from B-1 (expression of CD5) and B-2 cells (high expression of CD1d, like the marginal zone B cells), has recently been reported to play a critical role in the regulation of murine EAE \(^{82}\). However, the B cell subsets involved in suppression of other experimental immune diseases do not have exactly the same phenotype \(^{81,191-94}\).

Ambiguity therefore remains regarding the B-cell subpopulation(s) involved in the regulation of immune responses. An alternative hypothesis is that the immune suppressive activity is not a unique property of a single B cell subset but is perhaps exerted by different B cell subsets, depending on the integration of available signals in the microenvironment.

**Clinical implications**

Collectively, these studies demonstrate that Janus-faced B cells play both pathogenic and regulatory activities in immunopathogenesis (Figure 1) and that these opposing contributions may overlap during the course of the disease. Consequently, the therapeutic effect of B cell depletion depends on the relative contributions of the opposing B cell activities at the time of drug administration (Figure 2). It would be of utmost importance to define simple criteria or reliable markers that would help to predict whether administration of rituximab would be beneficial for the patient or not. Although there are clues about directions that should be taken in the future, there is no easy answer to this question yet. The recent identification of a human B cell subset with regulatory properties in the peripheral blood of lupus patients \(^{86}\) represents an
important step but will likely be insufficient since this subset appears to be qualitatively rather than quantitatively deficient in these patients. A functional assay, that would measure the response of T cells to a normalized stimulation in presence or absence of the B cells could be theoretically useful but its development would require an important amount of work, the limitations of functional assay for routine immunomonitoring being well known (minor changes of test conditions potentially having a major impact on the test results, necessity to work with freshly-isolated cells...etc). Of note, immune stimulation resulting from the removal of B cell regulation could also be used in a therapeutic perspective. This strategy could be particularly useful in cancer, where B cells have been shown to inhibit the induction of T cell-dependent protective anti-tumor immunity. Accordingly, the administration of anti-CD20 antibodies slowed the growth of non-hematopoietic solid tumors (not expressing CD20), and enhanced the efficiency of a tumor vaccine in a murine model. It is thus conceivable that the mode of action of Rituximab in hematological malignancies also relies in part on such a mechanism (Figure 1, lower panel).

**Conclusions**

The optimization of B cell depletion strategies for the treatment of immune diseases should therefore not only focus on the identification of new targets, nor on the development of more depleting drugs, but also requires a better understanding of the complex temporal interplay between pathogenic and regulatory B cells. Instead of a mere depletion of the B-cell compartment, future therapy should instead attempt to reset the regulatory balance - to which B cells can clearly contribute.
Acknowledgments

This work was supported by grants from the CENTAURE Transplantation Research Network and the Hospices Civils de Lyon.

Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interest.
Bibliography


60. Shaikh A, Habermann TM, Fidler ME, Kumar S, Leung N. Acute renal failure secondary to severe type I cryoglobulinemia following rituximab


Figure Legend

Figure 1

**Title: Mechanisms of action of anti-CD20 antibodies**

**Upper panel:** Apoptosis of malignant B cell clones occurs upon cross-linking of rituximab-CD20 complexes in the lipid rafts. This activates signaling pathways involving the Src kinases and their regulatory molecules. Complement-mediated cytolysis involves the ability of anti-CD20 IgG1 bound to their antigen to bind C1 and trigger the classical complement pathway. Antibody-dependant cell cytotoxicity requires interaction between the Fc portion of rituximab and appropriate receptors on effector cells.

**Lower panel:** Another possible mechanism by which rituximab could promote the destruction of malignant B cell clones is the restoration of the protective anti-tumoral response. The destruction of the non-malignant B cells endowed with immune regulatory properties could facilitate the development of anti-tumoral T-cell clones.

Figure 2

**Title: Janus-faced B cells**

Janus, the Roman god of beginnings and endings, is most often depicted as having two faces facing opposite directions. Like him, B cells can either elicit (right panel) or terminate (left panel) an immune response, following their activation by combination of ligation of the B-cell receptor, CD40, and toll-like receptors (TLR).
**Left panel:** B cells present the antigen along with costimulatory signals, leading to the activation and the proliferation of T effectors. In turn, activated T cells provide CD40L for the differentiation of B cells into antibody producing plasma cells. The cytokines produced by B cells may participate into the polarization of T effectors. Finally B cells play a critical role for the development of T cell memory.

**Right panel:** B cells regulate immune response by provision of IL10 that suppresses the activation and the expansion of T effectors directly, and indirectly through the differentiation of T reg and the suppression of dendritic cell function.

**Figure 3**

**Title: B cell depletion has ambivalent effects on the course of immune diseases**

The figure is a schematic representation of the findings of Matsushita et al. During the course of EAE, B cells play opposite overlapping roles. Depending on the timing of anti-CD20 antibody administration, the net clinical effect can be deleterious, neutral, or beneficial. Dashed lines indicate the severity of EAE in untreated control animals.
Figure 1

Complement-mediated cytolysis

Induction of apoptosis

B cell clone

Antibody-dependant cell cytotoxicity

Fc receptor
NK Macrophages Granulocytes

Legends
CD20 antigen
Anti-CD20 Ab

(anti-tumoral response)
Figure 3

B cell contribution to the disease

Timing of B cell depletion

Disease severity

Regulation Pathogenesis

\[ \text{Time} \]

\[ \text{Disease induction} \]
Am''B''valent: anti-CD20 antibodies unravel the dual role of B cells in immunopathogenesis

Olivier Thaunat, Emmanuel Morelon and Thierry Defrance