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Rituximab: Beyond Simple B Cell Depletion

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Abstract Rituximab, a chimeric anti-CD20 monoclonal antibody, has a proven track record for over a decade in the treatment of lymphomas, where it has been used to eradicate malignant lymphocytes. In appreciation of the putative role of B cells, especially with respect to autoantibody production, in the pathogenesis of autoimmune diseases, successful trials of B-cell depletion therapy in RA, SLE, and other autoimmune diseases have been carried out. In these trials, clinical benefit has generally correlated with the extent and duration of B-cell depletion, but at times imperfectly, and autoantibody reduction only selectively. Additional mechanisms whereby rituximab may assert its clinical benefit in autoimmune diseases have been examined including a look at B-cell functions as T-cell modulator and antigen-presenting cell, T-regulatory cell behavior, NK cell activity, and macrophage activities in immune inflammation. The available data on rituximab's action in autoimmune diseases is reviewed.

Keywords Rituximab · B-cell depletion · Lymphomas

Introduction

Rituximab is an IgG1 chimeric murine-human monoclonal antibody to CD20 which induces depletion of B cells [1]. CD20 is a highly specific surface antigen that is expressed on pre-B and mature B cells, but is not found on precursors or plasma cells. Several potential mechanisms for rituximab-induced B-cell depletion (Fig. 1) have been described. These include antibody-dependent cellular cytotoxicity (ADCC) through recruitment of natural killer (NK) cells, macrophages, and monocytes by way of CD20-bound Fcγ receptors leading to B-cell apoptosis; complement-mediated lysis by binding of C1q to rituximab bound to cell surface CD20 on B cells with resultant generation of membrane attack complex; and finally, the direct disruption of signaling pathways so as to trigger pro-apoptotic signals via CD20 on B cells. The contribution of each mechanism to the rituximab-induced depletion of B cells varies in different diseases [2, 3]. In 1997, rituximab became the first monoclonal antibody approved for the treatment of B-cell malignancies, making use of its efficient ability to induce B-cell depletion so as eradicate the cancerous lymphocytes. Currently, it is approved for the treatment of relapsed or refractory, low-grade or follicular, CD20+ B-cell lymphomas [4].

A significant role for B cells in the pathogenesis of autoimmune diseases was recognized following the landmark study by Shlomchik et al. showing that systemic lupus erythematosus (SLE)-prone MRL-lpr/lpr mice lacking B cells do not develop SLE–nephritis nor autoantibodies, thus putting forward these cells as potential targets in the therapy of autoimmune diseases [5]. At present, the role B cells play in human SLE and other autoimmune diseases is more fully appreciated as many of the clinical manifestations, such as hemolytic anemia and glomerulonephritis, appear to be antibody mediated. But apart from being autoantibody producers, B cells also produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, as well as interleukin (IL)-4 and IL-10, and are thus potential regulators of other effector cells, functioning as modulators of inflammation [6, 7]. Finally, B cells are superb and efficient antigen-presenting cells (APCs), as they

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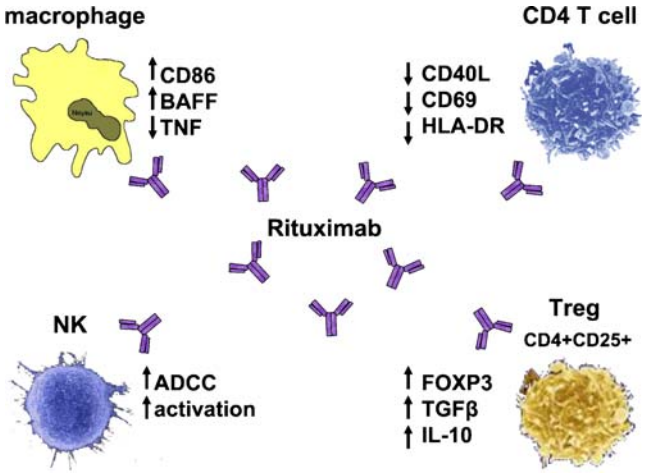

Fig. 1 Rituximab—beyond B-cell depletion mechanisms: 1. Rituximab decreases CD4 effector cell activation by reducing HLA-DR, CD69, and CD40L expression on T cells. 2. It reduces NK cell activation. 3. It increases the suppressive abilities of T-regulatory cells. 4. Rituximab induces macrophage maturation and reduces TNF secretion

can express MHC class II and co-stimulatory molecules such as CD80 and CD86 [8].

Appreciation of the above set the stage for the successful use of rituximab in the treatment of autoimmune diseases, initially rheumatoid arthritis (RA [9]), with the aim of inducing depletion of B cells, especially plasma-cell precursors or memory B cells becoming antibody producers as a means of reducing autoantibody production and modulating immune inflammation. Later on, an open study on SLE patients demonstrated rituximab treatment to be safe and effective in this setting as well, with improvement of disease manifestations [10]. These results were then confirmed by further reports of good clinical responses of patients with rheumatoid arthritis, polymyositis/dermatomyositis, idiopathic thrombocytopenic purpura, Wegener's granulomatosis, and SLE to rituximab [11, 12]. In a subsequent study, 43 patients were analyzed: 14 with RA, 13 with SLE, 6 with primary Sjogren's syndrome (pSS), 5 with systemic vasculitis, and 5 with other autoimmune diseases. In all cases, rituximab was administered to subjects with autoimmune disease refractory to conventional therapy. Efficacy was observed in 30 patients (70%): RA 11; SLE 9; pSS 5; and five with other autoimmune diseases. The mean decrease in corticosteroid intake of responding patients was 9.5 mg/day, a measure of the beneficial effect of rituximab in these cases [13].

Following the successful pilot study on B-cell depletion therapy in RA, a randomized, double-blind, controlled study including 161 patients who had active RA despite treatment with methotrexate (MTX) was conducted [14]. Responses were assessed at weeks 24 and 48. In this study, the addition of rituximab to MTX appeared to reduce the signs and symptoms of RA significantly, as assessed by ACR 20, 50, and 70 response criteria, and to be relatively safe. At week 24, the proportion of patients with 50% improvement in disease symptoms, was significantly greater with the rituximab–MTX combination (43%, $p=0.005$) than with MTX alone (13%). All ACR responses were maintained at week 48 in the rituximab–MTX group.

To study the quantitative and phenotypic reconstitution of peripheral blood B cells and its relationship to the dynamics of clinical response in patients with RA after B-cell depletion with rituximab, 24 patients with active RA were assessed [15]. The frequency and total number of CD19+ cells in the peripheral blood decreased a mean of 97% for more than 3 months in all patients. All B-cell populations were depleted. More than 80% of residual B cells showed a memory or plasma cell precursor phenotype. B-cell repopulation occurred during a mean of 8 months after treatment and was dependent on the formation of naïve B cells, which showed an increased expression of CD38 and CD5. During repopulation, increased numbers of circulating immature B cells were identified. Patients who experienced a relapse of RA on return of B cells tended to show repopulation with higher numbers of memory B cells. This suggests that RA remission is associated with a profound depletion of all peripheral blood B cell populations and that repopulation occur mainly with naïve mature and immature B cells.

To assess the clinical and basic serological consequences of B-cell depletion in the treatment of patients with SLE who failed conventional immunosuppressive therapy, 24 patients with severe SLE, followed for a minimum of 3 months, were studied [16]. Disease activity, anti-dsDNA antibodies, and serum C3 levels were studied during the entire follow-up period. Disease activity score ($p<0.0001$), serum C3 ($p<0.0005$), and dsDNA binding ($p<0.002$) all improved from B-cell depletion to 6 months after this treatment. On monitoring, the time period that B-lymphocytes remained depleted ranged from 3 to 8 months, except for one patient who remained depleted for more than 4 years. The mean daily prednisolone dose fell from 13.8 to 10 mg.

In parallel, the efficacy of rituximab in the treatment of lupus nephritis was also evaluated. Patients with active proliferative nephritis, four with focal disease and six with diffuse disease, received rituximab—4 weekly infusions of 375 mg/m^2 combined with oral prednisolone [17]. Clinical, laboratory, and immunologic responses, including peripheral lymphocyte subsets, were prospectively assessed at monthly intervals for 12 months. Complete remission of nephritis was defined in the presence of normal serum creatinine and albumin levels, inactive urine sediment, and 24-h urinary protein <500 mg. B-cell depletion lasted from 1 to 7 months and was well tolerated. Partial remission was achieved in eight of ten patients within a median of 2 months; in five of them, complete remission was subsequently established at a median of 3 months from baseline and sustained for12 months in four patients.

As part of a phase I/II dose-ranging trial of rituximab in the treatment of SLE, the fate of discrete B-cell subsets in the setting of selective depletion by the anti-CD20 monoclonal antibody and during the B-cell recovery phase was evaluated [18]. Compared with normal controls, SLE patients displayed several abnormalities in peripheral B-cell homeostasis at baseline, including naïve lymphopenia, expansion of CD27-, IgD-, and expansion of lymphoblasts. These abnormalities resolved after effective B-cell depletion. The frequency of autoreactive VH4.34 memory B cells also decreased 1 year posttreatment, despite the presence of low levels of residual memory B cells at the point of maximal B-cell depletion and persistence of elevated serum autoantibodies in most patients. This persistence of elevated autoantibody titers may reflect the presence of low levels of residual autoreactive memory B cells and/or long lived autoreactive plasma cells.

The long-term efficacy and safety of rituximab in patients with SLE and patients with vasculitis, 11 with active or refractory SLE and 11 with active or refractory antineutrophil cytoplasmic antibody-associated vasculitis (AAV), was further assessed in a prospective study with a median follow-up of 24 months [19]. Remission followed rapid B-cell depletion, with response rates of 100% among the 11 patients with SLE, 6 patients with complete response and 5 patients with partial response, and 91% among the 11 patients with AVV, 9 patients with complete response and 1 patient with a partial remission. All six patients with lupus nephritis demonstrated a good clinical renal response. Clinical improvement was accompanied by significant reductions in the daily dose of prednisolone. Relapse occurred in 64% of the patients with SLE and in 60% of those with AAV. B-cell return preceded relapse in the majority of patients. Repeat treatment with rituximab proved effective. The above results in aggregate provide strong support for the performance of a double-blind controlled trial in SLE as well.

Thus, from the studies reported, it appears that B-cell depletion offers the prospect of sustained disease remission and improved disease control in many autoimmune diseases. Relapse after treatment is common, but re-treatment is safe and rapidly effective.

B-Cell Depletion and Autoantibodies

While rituximab's clinical effect in autoimmune diseases have been impressive, the mechanism by which this is achieved are not entirely clear. In early studies of rituximab's effect in autoimmunity, the changes in serologic variables correlating with clinical disease activity in RA, after B-cell depletion, were assessed [20]. Levels of C-reactive protein (CRP), of autoantibodies including IgM and IgG-class rheumatoid factor (RF) and of antibodies to cyclic citrullinated peptide (anti-CCP) were analyzed. The majority of patients showed marked clinical improvement after treatment with rituximab, with benefit lasting up to 33 months. Both RF and anti-CCP antibodies decreased significantly, even more than did those of their corresponding total serum immunoglobulin classes. The kinetics for the reduction in CRP levels also paralleled the decreases in autoantibody levels. B lymphocyte return occurred up to 21 months posttreatment, but the time to relapse after B lymphocyte return was often long and unpredictable (range 0–17 months). Relapse, however, closely correlated with rises in the level of at least one autoantibody. Increased autoantibody levels were rarely observed in the absence of clinical change.

The issue of whether clinical benefit in both SLE and RA is always closely correlated with the extent of B-cell depletion and whether remission is associated with a decrease of all relevant autoantibodies is still pending, however. In this connection, a total of 100 rituximab-treated patients with severe SLE, refractory to major immunosuppressive treatment, were reviewed [21]. With a median follow-up period of 12 months, rituximab was well tolerated, compatible with the experience accumulated from its use in more than 500,000 lymphoma patients. About 80% of the patients achieved marked and rapid reduction in global disease activity. Because of the clinical heterogeneity of the patients, dosing differences, and varying concomitant treatments, including cyclophosphamide in 35% of patients, a proper evaluation of the clinical efficacy or rituximab was difficult. Variable degrees of clinical benefit were reported for all clinical SLE manifestations, including active proliferative nephritis. Whereas four weekly infusions of 375 mg/m of rituximab resulted in complete B-cell depletion lasting most often from 3 to 8 months, a prolonged depletion did not always correlate with a more favorable clinical response. Total immunoglobulin levels and protective antibodies were preserved, but anti-dsDNA antibody titer decreases were often found to be independent of the clinical response, raising the possibility that rituximab may affect other distinct autoimmune pathogenic processes.

In this regard, other studies have shown that clinical benefits after rituximab initiation can still persist despite of abrogation of B-cell depletion [22]. Although normalization of anti-dsDNA antibodies and complement levels correlated with other measurements in the responders, these changes did not appear in some of the responders and were found, on the other hand, in some patients considered as treatment failures [15]. These inconsistent results require reassessment and raise the possibility that rituximab could modulate other immune functions apart from those that are autoantibody related.

Studies on rituximab's effect on B cells, the decline in B-cell number, only moderate reduction in immunoglobulin levels, and specific effects on antibodies associated with RA were repeated. In one study, peripheral blood B-cell depletion was achieved 1–2 months after rituximab therapy in 15 RA patients [23]. In parallel with the beneficial clinical outcome, serum levels of RF declined 1–2 months post-B-cell depletion therapy and remained so to 3–4 months post-depletion therapy. Elevated anti-CCP antibodies persisted during the follow-up, however, and did not decline regardless of B-cell depletion and/or beneficial outcome.

Similar results were found again in our recent study [24]. After rituximab therapy, RF disappearance or titer decline occurred in six out of seven RA patients, in positive correlation with clinical remission. Again, anti-CCP antibodies remained unchanged in six RA patients despite documented beneficial response. To the extent that anti-CCP is a more specific and hence "truer" reflection of the RA disease process, discordance between the B-cell depletion documented in the present study and continued presence of anti-CCP, also a B-cell product, is consistent with the understanding that B-cell depletion by itself is not responsible for the clinical improvement in patients treated with rituximab. Furthermore, the general observation that many RA patients experience relapse of their clinical disease sometime between 6–12 months after B-cell depletion, when B-cell count is still quite low, may suggest that the disease process persists with survival of some pathogenic memory B cells, possibly producers of pathogenic autoantibodies such as anti-CCP antibodies, that had escaped depletion and kept expanding in secondary lymphoid tissues [20].

Thus, the inadequacy of viewing rituximab's beneficial clinical effects as the product of simple B-cell number reduction has motivated many to focus on T cells and macrophage functions as additional effectors that play a role in the favorable outcome of rituximab treatment in autoimmune diseases.

Mechanisms Beyond B Cells

Although CD20 is expressed mostly on B cells at various stages of development, a small number of T cells and NK cells also express low levels of CD20. As a consequence, the numbers of T and NK cells have been shown to be significantly reduced during rituximab treatment in RA patients [15]. Repopulation of these cells was shown to occur after a mean of 5 months after treatment. Therefore, it is to be expected that rituximab, via its effect on cell numbers, may also exhibit some direct effect on T-cell immunity.

T Cells Of relevance, B cells in both SLE and RA display abnormal signaling, expressing aberrant cell-surface markers (e.g., CD40 ligand), thereby activating T cells through cognate interactions and helping organize and regulate inflammatory immune responses [17]. This suggests an additional path for B cells, independent of autoantibody production, to play a role in promoting these diseases. In this respect, it has been demonstrated that rituximab treatment not only reduces B-cell number and IgG levels but also down-regulates CD40 and CD80 on B cells, affecting a possible disturbance of T-cell activation through these co-stimulatory molecules [25]. As early as 1 month after the initiation of rituximab treatment for lupus nephritis, the expression of the co-stimulatory molecule CD40 ligand on CD4+ T cells was decreased by fourfold, and nearly totally blocked when partial remission was clinically evident [17]. Also the expression of T-cell activation markers CD69 and HLA-DR was significantly decreased at time points when partial remission was observed, with further decrease during complete remission. Thus, after B-cell depletion, clinical remission of lupus nephritis is also associated with a decrease in T-helper-cell activation. These findings point to the issue of rituximab influence on T-cell activation and responses as part of its mechanism of action in ameliorating autoimmune disease.

T-Regulatory Cells The function of T-regulatory cells was investigated in a study where 22 patients with active SLE and renal involvement, refractory to conventional therapy, were treated with rituximab [26]. The levels and function of regulatory T lymphocytes and the apoptosis of immune cells were assessed. Clinically, there was a significant reduction in disease activity, as what was manifested in decreased proteinuria, erythrocyturia, and improved creatinine clearance. In 20 of 22 patients, B-cell depletion was observed, but no clear-cut effect of rituximab on complement levels or autoantibody titers was detected, $p>0.05$ in all cases. A significant enhancement in the levels of different CD4+ regulatory cells (Treg, Th3, Tr1), but not CD8+ Ts lymphocytes, was observed at day 30. This increase was sustained for Treg cells at day 90 and was accompanied by improvement in their regulatory function. In most cases, this apparently beneficial effect was affected by increased suppressive function of the following T-regulatory cells: Treg (CD4+ CD25bright) lymphocytes, Tr1 (CD4+ IL-10+) cells, and Th3 (CD4+ TGF-β+) lymphocytes. The investigators speculated that the decrease of antigen-presenting cells induced by rituximab induced a

decrease in activated T cells, which express, among other cell markers, the ligand of the glucocorticoid-induced TNF receptor (GITRL). In view of the fact that it has been described that GITRL is able to inhibit the anergic behavior and suppressive function of Treg, it is feasible that a decrease in lymphocytes bearing GITRL favors an increase in the number and function of regulatory cells.

In a most recent study, leukocyte subpopulations and antibody titers were investigated in rituximab-treated SLE patients [27]. While the few B cells remaining after treatment were of memory, double-negative (IgD−CD27−), and CD5+ phenotype, the returning B cells were mainly naïve, indicating de novo production of B cells. Serum levels of IgG and antibodies against Ro52, Ro60, La44, measles, and tetanus remained unchanged, while decreases in IgM, IgE, anti-dsDNA, and anti-C1q antibodies were observed. Of note is the observed significant increase in CD25bright FOXP3+ regulatory T cells after B-cell depletion, indicating that both humoral and the cellular immune systems were affected by treatment with rituximab. In addition, an unexpected increase in the apoptosis of T cells at day 30 was observed and was attributed to IL-10 reduction, after B-cell depletion, contributing to the stimulation of expanded T-cell subsets in SLE.

NK Cells Changes in NK, macrophage, and or dendritic cell functions have also been reported during B-cell depletion. The sensitivity of freshly isolated neoplastic B cells to rituximab-mediated ADCC was studied [28]. ADCC was performed by 51Cr release assays in vitro, using peripheral blood mononuclear cells, IL-2-activated or expanded NK cells, neutrophils or macrophages as effector cells. Purified NK cells (95% CD56+/CD16+) reached 70% lysis at the highest effector/target ratio. Interestingly, short-term IL-2 cultured PBMC, containing 10% activated NK cells and long-term expanded NK cells, containing 80–95% activated NK cells, became strong ADCC effector cells with rituximab and lysed all leukemic samples to a mean of 57% and 67%, respectively. Thus, short-term exposure to IL-2 or long-term expansion of NK cells in vitro may provide effective tools to improve the therapeutic activity of rituximab in autoimmune diseases as well.

Macrophages In another study, ex vivo-activated human macrophages were shown to kill chronic lymphocytic leukemia cells in the presence of rituximab [29]. These macrophages synergize with the anti-CD20 Ab rituximab for killing primary B-cell chronic lymphocytic leukemia (B-CLL) cells. ADCC reached levels of 70% to 80% at effector to target ratios as low as 1:1. Macrophage recruitment by Ab-opsonized tumor cells did not result in enhanced cytokine secretion, suggesting that the cytokine shower observed in rituximab-treated patients is not caused by macrophage activation and that cytokines have no role in CLL killing. Uptake of tumor material by macrophages did not directly correlate with tumor killing. Nonetheless, experiments in the presence of cytochalasin D showed that ADCC occurred mainly by phagocytosis. Tumor killing was largely mediated by Fc gammaRI and inhibited by increasing concentration of serum. Again, although the above studies refer to tumor killing, they point to an effect of rituximab directly on macrophages, another mechanism of possible importance in therapy of autoimmune diseases.

Evidence for macrophage function alteration by rituximab therapy in RA patients was presented in our recent study [24]. In this study, we reported on the association between rituximab-related cell depletion and increased B-cell activating factor (BAFF) and IL-10 mRNA expression in macrophages of patients with RA at baseline and 4 months after rituximab initiation. The increase in mRNA BAFF expression in macrophages was thought to be a compensatory effect that may be responsible for B-cell repopulation and survival. The finding of increased IL-10 mRNA in macrophages of RA patients after rituximab-induced B-cell depletion may be a part of wider immunomodulation, including anti-inflammatory changes, in macrophage function in this setting. In addition, there was significant reduction in TNF-α in the supernatant of cultured macrophages. As generally immature macrophages are major TNF-producers, dominant during active RA, possibly the macrophage population post-B-cell depletion is replaced by a different, possibly more mature, macrophage population of less pro-inflammatory character.

Depletion of B lymphocytes which function as APCs with rituximab may drive the need for mature macrophages that also function as APCs, to maintain a normal protective immune response. In our study, increased mRNA CD86 expression in macrophages was also documented, compatible with such a response of mature macrophage-APCs contributing to the maintenance of a protective immune response and relative lack of infections, despite B-cell depletion.

In summary, the above results reveal the complexity of mechanisms that underline the beneficial results of rituximab therapy in autoimmune diseases. More data suggest that by increasing suppressive functions of T-regulatory cells, rituximab could lead to the attenuation of pro-inflammatory CD4+ T-cell responses. Finally, rituximab also appears to affect NK cells' ADCC function and to decrease macrophage activation. When and in which situation each of these mechanisms is dominant should be the subject of many future studies. Further study is to be

encouraged so as to ferret out and better understand the intricacy of mechanisms whereby rituximab exerts its effect, mainly those that go beyond simple B-cell depletion.

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