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B cell depletion therapy in autoimmune diseases

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1. ABSTRACT

Our understanding of the multiple physiological and pathological functions of B-cells continues to expand at a fascinating rate. A critical part of this expanding knowledge is the realization that B-cells can be responsible, at least in part, for diseases in which they had not been previously suspected and that their pathogenic influence can be mediated by multiple mechanisms. In turn, the availability of effective agents capable of inducing profound and long-lasting B-cell depletion and the safety and efficacy of Rituximab in non-Hodgkin lymphoma has prompted investigators to use this therapeutic approach in a large number of autoimmune diseases. Thus far, the results have been very promising, and in some cases nothing short of spectacular. In this review, we shall discuss the roles of B-cells in health and disease and the available evidence regarding the efficacy of B-cell depletion in human autoimmunity. Finally, we will discuss some of the many challenges and opportunities that the medical and scientific community should address in the foreseeable future.

2. INTRODUCTION

The last decade has witnessed an explosion of biological approaches to medical therapy in general and to the treatment of autoimmune diseases in particular. This revolution is perhaps best exemplified by the dramatic impact that agents capable of blocking Tumor Necrosis Factor (TNF) have had in the treatment of patients with Rheumatoid Arthritis (RA). A critical aspect of biological therapies is that they provide medical scientists with a unique, albeit often under exploited, opportunity to understand disease pathogenesis and to determine by which mechanisms these interventions actually work. This situation is powerfully illustrated by strong evidence of clinical benefit induced by the elimination of B cells in an ever growing number of autoimmune diseases including some in which B cells had not been previously convicted or even indicted as the main nefarious agents. In this review we shall discuss the most relevant biological and pathogenic functions of B cells, the therapeutic benefit of B-cell depletion and the challenges and opportunities created by this type of intervention.

3. PATHO-PHYSIOLOGICAL FUNCTIONS OF B CELLS

Even today, antibody production is still widely considered the main or only significant function of B cells, at least outside the world of B-cell aficionados. It is obviously true that antibody production is unique to B cells and stands in contrast to antigen presentation or cytokine production, functions which are shared by multiple cell types, including B cells themselves. Moreover, there is overwhelming evidence that autoantibodies contribute to autoimmunity by multiple mechanisms, including immune-complex mediated type III hypersensitivity reactions, type II antibody-dependent cytotoxicity, and by instructing innate immune cells to produce pathogenic cytokines including IFNα, TNF and IL-1 (1-3). Nevertheless, this antibody-dominated view of B cells flies in the face of abundant evidence for multiple other functions of great physiological and pathological relevance (1, 4).

While different activities have been ascribed to B cells over the years and interesting phenotypes have been described in B-cell deficient mice, it has been harder to separate the effects of antibody production from other B-cell functions. More recently the pioneering work of Shlomchik's group, using mice in which B cells have been rendered unable to secrete antibody, has demonstrated the ability of B cells to participate in disease pathogenesis by antibody-independent mechanisms (5-9). Subsequently, this notion has been corroborated in humans by our own findings that clinical improvement in SLE patients treated with Rituximab correlates with B-cell depletion and precedes by several months any decline in serum levels of relevant autoantibodies (10).

4. ANTIBODY-INDEPENDENT B-CELL FUNCTIONS

4.1. Lymphotoxin-mediated effects

Many antibody-independent functions demonstrated for B cells are mediated by lymphotoxin-α (LTα). Indeed, B cells, which are the main producers of LTα, play fundamental roles in the organization of the lymphoid architecture and in the orchestration of critical cellular interactions, to a large extent through the production of LTα1β2 (signaling through LTβR) and TNF (signaling through the TNFR1) (11, 12), thereby controlling: i) the formation of the T cell zone; ii) the formation of the marginal zone; iii) the maturation of follicular dendritic cells (FDC) networks; and iv) the development and structure of the germinal centers (11-17). LTα1β2 signaling also induces production of CCL19, CCL21 and CXCL13 in stromal cells and is critical for the homeostasis of lymphoid tissue dendritic cells (DCs) and for the recruitment of a specific subset of germinal center dendritic cells (CR-Fc$^+$ dendritic cells), which are characterized by expression of the cysteine-rich domain of the mannose receptor (11, 18-24). These effects have been demonstrated in different animal models, including B-cell deficient mice, which are characterized by profound defects in T cell formation and decreased numbers of T cells and DC (19, 22, 25).

Of particular significance for autoimmunity, LTα1β2 signaling is also central to lymphoid neogenesis, or tertiary lymphoid tissue formation, a process that may lead to dysregulated B/T-cell interactions, ectopic germinal center formation and local amplification of autoimmune responses in multiple autoimmune diseases including rheumatoid arthritis, Sjogren's syndrome, SLE, type 1 diabetes, multiple sclerosis, myasthenia gravis, hepatitis C, inflammatory bowel disease, autoimmune gastritis, primary sclerosing cholangitis and Hashimoto's thyroiditis (20, 26-42). A particularly provocative example of the central participation of B cells in the pathological process taking place in tertiary lymphoid tissue is illustrated by the demonstration that CD4$^+$ T-cell activation in the rheumatoid synovium is dependent on the presence of B-cell follicles and that the depletion of B cells in this model inhibits the T-cell production of IFNγ and IL-1 (43).

4.2. T-cell effects

One central mechanism whereby B cells regulate T cells is through antigen-presentation. This function can be mediated by antigen-specific B cells and at least as efficiently, by rheumatoid factor (RF)-producing B cells which can present virtually any antigen owing to their ability to capture immune complexes (44). Obviously, the latter mechanism may be of particular significance for RA or other entities characterized by excessive production of RF (i.e., Sjögren's syndrome or hepatitis C).

The following effects of B cells on T cells have been described:

Priming and clonal expansion of naïve CD4$^+$ T cells. Although naïve T-cell responses are normally primed by dendritic cells, B cells are also very efficient antigen-presenting cells (APC) and have been postulated to play important roles in the initial T cell priming (45-50). While not fully understood, B cells are also likely to play important roles in the reactivation of memory responses, and the continued stimulation and expansion of primary responses may be assisted by activated B cells (50-54).

Priming of naïve CD8+ T cells which results from DC activation induced by TACI-BLyS interaction (55).

Activation of autoreactive CD4$^+$ and CD8$^+$ T cells by activated B cells (5, 43, 56-58).

Promotion of Th2 differentiation through co-stimulation by B-cell borne OX40L and by B-cell derived IL-10 (52, 59-65) (66). In this regard, it is important to note that in murine models of SLE, a restricted diversity of B cells inhibits hyperactivation of Th1 cells and substantially prolonged survival, and that similar Th1 inhibition has been postulated in human SLE patients treated with Rituximab (58, 67). These effects however could be balanced by the ability of B cells to induce Th1 differentiation through the production of IL-12p70 (68).

Recruitment/co-localization in the germinal centers and modulation of the helper phenotype of $CXCR5^+$ follicular T Helper (F_{TH}) cells (65, 69). F_{TH} cells provide critical assistance for follicular and germinal center B cells and induce activation, differentiation and antibody production, at least in part through ICOS-ICOS-L interactions and secretion of IL-21 (65, 70-74). It is important to emphasize the influence of B cells on T_{FH} cells since excessive activity of F_{TH} has been shown to result in hyperactive germinal centers, systemic autoimmunity and Lupus-like disease (75).

Inhibition of expansion of regulatory (Treg) cells. Of significant interest, the ability of B-cells to block regulatory T cells has been implicated in the development of Crohn's disease and autoimmune thyroiditis in mice (76, 77).

4.3. Dendritic cell effects

As previously indicated, B cells have the potential to modulate DC maturation, migration and recruitment through the production of LTα and TNFα. B cells can also influence the migration and maturation of DCs through the production of IL-16 and other survival and chemoattractant factors including MIP-1α and MIP-1β (19, 78-80).

Overall, B cells appear to promote induce activation of DC at least in part through TACI-induced upregulation of CD86 (55). This B cell function may be important for priming of CD8+ T cells and may help explain important antibody-independent roles of B cells in viral immunity (81-85). A recently described novel function of B cells is worth discussing in this context. B cells appear to be responsible for lymphangiogenesis and lymph node expansion in response to immunization. In turn, the expanded lymphatic network substantially enhances DC mobilization and migration to the draining nodes, thereby allowing the priming of T-cell responses. Interestingly, this study demonstrated that lymph node B cells prominently express VEGF-A (vascular endothelial growth factor-A) and postulated the involvement of this lymphangiogenic growth factor in the lymph node growth and DC migration observed (86). It should be noted however that contrasting effects on DC activation such as the ability of activated lymph node B cells to inhibit dendritic cell induction of T-cell immunity have also been described (87).

In addition, B cells may also profoundly influence the antigen-presenting activity of DCs both in vitro and in vivo. Specifically, B cells may dampen IL-12-induced Th1 differentiation promoted by DCs. This effect appears to be mediated by IL-10 and possibly by IL-6 (59, 60, 63).

4.4. Cytokine production

B cells are the main producers of LTα and an important producer of TNFα, both cytokines that as previously discussed play central roles in lymphoid development and organization. In addition, an often underestimated aspect of B-cell biology is the ability of human B-cells to secrete multiple other cytokines which has been nonetheless recognized for many years (88). These cytokines include IL-1, IL-4, IL-6, IL-8, IL-7, G-CSF, GM-CSF, IL-10, IL-12 and TGFβ (54, 88, 89). More recently, Frances Lund and co-workers have elegantly demonstrated that effector B-cells (Be1 and Be2) can be induced to produce cytokines in polarized patterns largely mimicking those of Th1/Th2 cells. Furthermore, Be1 and Be2 cells can participate in feedback regulation of T helper cells (90, 91).

The functional significance of cytokine production by B cells is also supported by the ability of B-cell derived IL-10 to inhibit Th1 priming typically induced by DCs, as discussed above. This type of B-cell activity may be important in vivo as it has been postulated to influence Th1/Th2 balance during development and in the immune response to infections (92).

From an autoimmunity standpoint, cytokine production by B cells represents a double-faced Janus entity, for it may either stimulate or inhibit pathogenic responses. On one hand, B cells are able to suppress autoimmunity in different animal models, either through the production of IL-10 or TGFβ as well as by cytokine-independent functions (93-98). Collectively, these observation are starting to coalesce into the important concept of regulatory B cells (Breg) (99). The implications of this concept for therapeutic B-cell depletion will be further entertained at the end of this review. On the other hand, B-cell derived cytokines can play pathogenic roles in autoimmune diseases. This potential is illustrated by the increased production of IL-10 by B cells from patients with lupus and the improvement experienced by lupus patients treated with anti-IL-10 antibodies (100, 101). Similarly, bone marrow B cells producing TGFβ superfamily member bone morphogenetic proteins-6 and -7 have been implicated in the development of destructive arthritis, suggesting an additional pathogenic mechanism for B-cells in RA (102). The participation of B-cell derived cytokines has also been postulated to be responsible for the induction of liver fibrosis in an animal model (103, 104). This finding should stimulate new research into the contribution of B cells to liver damage in patients with hepatitis C, an infection characterized by B-cell hyperactivity and hepatic infiltration by B cells. The ability of B cells to promote fibrosis is also tantalizing in view of the likely role of B cells in scleroderma and the reduction of skin fibrosis observed in tight skin mice treated with depleting anti-CD20 antibodies (105).

4.5. B-cell modulation of the innate immune system

As previously discussed, B cells can have a powerful influence over innate immune cells, including DCs, through cytokine production. Moreover, B cells also influence innate immune responses through the generation of specific types of autoantibodies, mostly via their interaction with co-stimulatory Fc receptors. This type of function and its pathogenic significance are illustrated by the ability of SLE anti-RNP and anti-DNA antibodies to form immune complexes with apoptotic cells and induce IFNα secretion by plasmacytoid dendritic cells (2, 106). Similarly, arthritogenic antibodies can activate mast cells

and induce RA-like disease in K/BxN mice, at least in part through the production of TNFα and IL-1 (3, 107).

5. THERAPEUTIC APPLICATION OF B-CELL DEPLETION IN HUMAN AUTOIMMUNE DISEASES

Different agents can deplete or modulate B-cell numbers or function. Most of these agents are currently undergoing formal testing in clinical trials or are under development. Important examples include BlyS inhibitors, anti-IL-6, anti-IL-10, anti-CD22 and type 1 interferon inhibitors (1, 105, 108). Interestingly, our own preliminary results indicate that anti-TNF agents may induce germinal center disruption and significant reduction in memory B cells in RA patients, suggesting that B-cell effects should be considered as a potential mechanism of action of these agents in RA (109).

However, this review will be restricted to direct B-cell depletion induced by anti-CD20 antibodies and more specifically Rituximab (Rituxan™, MabThera™). Rituximab is an anti-human CD20 mouse/human IgG1 chimeric monoclonal antibody that reacts with the extra-cellular portion of the tetraspanin CD20 antigen which is expressed by all B cells from the pro-B cell stage to the early plasmablast stage. CD20 expression is specific for the B-cell linage and accordingly, Rituximab exclusively kills B cells. While some early/immature plasma cells may express decreased amounts of surface CD20, by and large, mature plasma cells are devoid of CD20 and they are therefore impervious to treatment with Rituximab (110).

Several killing mechanisms have been demonstrated for Rituximab including CD20-induced apoptosis, complement-mediated cell lysis and antibody-dependent cytotoxicity (ADCC) (1, 110). In addition, at least in the treatment of lymphoma, a vaccinal effect has been postulated whereby B-cell killing would result in immunogenic presentation of immunoglobulin-derived peptides and elicit a cytotoxic T-cell response (111). However, the relative contribution of these mechanisms to B-cell killing in vivo has not been determined and is likely to be different for different diseases.

The ability of Rituximab to kill both normal and malignant B cells first observed in patients with follicular lymphoma has prompted the utilization of this modality for the treatment of an ever-expanding number of autoimmune diseases (112). To date Rituximab, which was initially approved by the FDA in 1999 for the treatment of follicular B-cell non-Hodgkin lymphoma, has been used to treat both systemic and localized autoimmune diseases affecting virtually any organ system (113) (114). Thus, rheumatic, hematological, neurological, dermatological, infectious (hepatitis C) and paraneoplastic autoimmune diseases have been treated with this agent (112). In addition, B-cell depletion is currently being studied in other autoimmune diseases prominently including Type 1 diabetes.

While in most cases only anecdotal evidence is available, the overall impression has been that Rituximab-induced B-cell depletion is both safe and generally effective. However, the basis and impetus for the use of Rituximab in autoimmune diseases stem from the impressive results obtained by different investigators in three different conditions (rheumatoid arthritis, systemic lupus erythematosus and Wegener's granulomatosis), which will be reviewed in more detail below. Promising results have also been reported in series of patients with idiopathic autoimmune thrombocytopenia, IgM-related polyneuropathies and dermatomyositis (115-120).

5.1. B-cell depletion in RA

Over time, different cell types have been favored as critical pathogenic players in RA. While B cells have been considered important as producers of rheumatoid factor and anti-citrullinated peptide antibodies as well as APC capable of presenting multiple antigens, it is fair to say that until recently they did not represent a major therapeutic target. Yet, a major pathogenic role for B cells has been suggested by the existence in the rheumatoid synovium of tertiary lymphoid tissue, in some cases with formation of ectopic germinal centers and abundance of plasma cells (32, 121). Moreover, B cells are needed to activate CD4+ T cells in the rheumatoid synovium and induce the production of TNFα, IL-1 and IFNγ (43). Initially based on the idea that RF-producing B cells could perpetuate themselves and induce production of TNFα, Edwards and Cambridge hypothesized that B-cell depletion could have a beneficial impact on patients with RA and treated 5 refractory cases with Rituximab in combination with cyclophosphamide and prednisolone (122). While the interpretation of results was confounded by the open label nature of the study and concomitant immunosupression, this study produced tantalizing results regarding the potential benefit of B-cell depletion. Convincing evidence was subsequently provided by the same group using a well-designed, randomized, double-blind, controlled study of 161 patients with active RA despite treatment with methotrexate. This study showed that when used in combination with either methotrexate or cyclophosphamide, a single course of two infusions of 1,000 mg each of Rituximab induced significant improvement both after 24 and 48 weeks as compared to patients who continued methotrexate (123). This study, however, did not definitively clarify whether corticosteroids were also required.

Just in the last year, two larger studies (REFLEX and DANCER) thus far published only in abstract form and presented at the 2005 annual American College of Rheumatology Symposium, have confirmed the efficacy and overall safety of this regimen (124-127). Furthermore, one of the studies also showed that the efficacy of the treatment was independent of the concomitant use of corticosteroids (128). Of great significance, the DANCER study also established the efficacy of Rituximab in RA patients refractory to anti-TNF therapy (126).

As a result of the combined evidence provided by the work just summarized, the FDA announced during the

writing of this manuscript (on March 1, 2006) the approval of Rituximab (in combination with methotrexate) for the treatment of RA resistant to anti-TNF therapy.

5.2. B-cell depletion in SLE

Evidence for the potential benefit of B-cell depletion in SLE has been gathered from the combination of the original dose escalation trials of Rituximab performed by our own group at the University of Rochester and by Albert and colleagues at the University of Pennsylvania, as well as from several open label series and numerous published and unpublished case reports (10, 129). For additional clinical details of these studies, the reader is referred to a recently published review (110).

Initial evidence for the efficacy of this therapy was offered by Leandro and colleagues, who reported on six refractory SLE patients treated with Rituximab in combination with cyclophosphamide and high-doses of oral corticosteroids. The same group has more recently reported similar benefit and safety profiles in 18 additional refractory patients similarly treated and followed longitudinally for up to several years (although follow-up in most cases was limited to 6 months) (130).

Our own phase I/II trial of 18 patients provided similar evidence for efficacy and safety, and patients enrolled in the study have now been followed for up to 5 years after treatment with Rituximab (10, 131). Patients were divided into three groups, the first two receiving single doses of Rituximab at either 100 mg/m^2 or 375 mg/m^2 and the third group receiving four infusions of 375 mg/m^2. In our study, Rituximab was added to ongoing therapy, which was generally milder (mostly with azathioprine and low-dose corticosteroids) and did not incorporate cyclophosphamide or additional steroids. Interestingly and in contrast to other studies of either SLE or RA, a significant fraction of the 17 patients evaluated did not experience profound B-cell depletion (defined as levels of peripheral blood CD19$^+$ B cells of < 5/μl). Poor depletion was seen in all three dosage groups and therefore was not exclusively due to low doses of Rituximab. In fact, our study demonstrated that poor depletion correlated instead with race (African-Americans), high-levels of serum human anti-chimeric antibodies (HACA) and the low-affinity allele of the FcγRIIIa (10, 132). Similar associations between good B-cell depletion and the high-affinity FcγRIIIa allele have also been reported in lymphoma patients treated with Rituximab (133, 134). These results are in keeping with recent observations in mouse models indicating that B-cell depletion is highly antibody isotype-dependent with IgG2a > IgG1 > IgG2b > IgG3. This gradient stems from the differential interaction of these isotypes with FcγRIV, FCγRIII and FcγRI. Interestingly, the highest and most dose-efficient degree of depletion was observed with anti-CD20 IgG2a antibodies through their interaction with mouse FcγRIV which is most structurally similar to human FcγRIII (135).

The presence of non-depletors in our study permitted us to determine whether there was a significant correlation between the degree of clinical response and the efficacy of B-cell depletion. Overall, good depletors experienced significant clinical improvement as measured by the SLAM index. As further discussed below, a subset of patients experienced complete clinical remission that has been sustained for the duration of follow-up (up to 5 years) in the absence of additional immunosuppression. Our study also established that initial and substantial clinical improvement can be generally achieved within the first 8 weeks after treatment, coincident with profound B-cell depletion and in the absence of significant decreases in autoantibody titers.

B cell depletion has also proven beneficial in studies of patients with specific types of lupus organ involvement such as renal and CNS disease (110). Thus, significant improvement in 15 patients refractory to conventional therapy, including 10 with active nephritis, has been reported by Van Vollenhoven and colleagues (136). This study provided histological evidence of improvement with repeat renal biopsies. Additional indication of the benefit of Rituximab in combination with significant doses of oral corticosteroids in 10 patients with proliferative lupus nephritis has been contributed by Sfikakis and colleagues who obtained a renal response in 8 patients (5 complete and 3 partial responses) (137). As regards CNS lupus, significant improvement was noted in two independent studies in patients with severe disease treated with Rituximab alone or in combination with cyclophosphamide (138, 139).

5.3. B-cell depletion in Wegener's granulomatosis

Severe Wegener's Granulomatosis is a life-threatening disease which resulted in almost universal demise of patients before aggressive treatment with cyclophosphamide was introduced and became a mandatory, life-saving part of the treatment. Growing experience with this approach however has exposed its significant shortcomings. Toxicity is substantial and prominently includes severe infections, hemorrhagic cystitis, ovarian failure and increased risk of malignancy. Furthermore, disease relapses are frequent upon discontinuation of therapy. Finally, approximately 10% of patients are refractory to treatment (140, 141). Accordingly, there is a great need in this severe disease for better and safer treatments, both for the induction and maintenance of remission as well as for the rescue of patients refractory to conventional treatment.

It is in this context that the original report of success with Rituximab for the treatment of 11 patients with refractory antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (10 of whom had Wegener's granulomatosis) has created great expectations (142). These patients had severe disease that created an immediate risk either to the patient's survival or to the function of a major organ. This report demonstrated the induction of disease remission in all patients, resulting in discontinuation of dialysis and ventilatory support in patients requiring these modalities, usually accompanied by significant decline or disappearance of ANCA within the first few months of treatment. The failure of ANCA to reappear in some patients upon B-cell reconstitution

suggests that B-cell tolerance could have been restored in these cases although additional, longer studies with detailed cellular studies will be required to establish this point. Limitations of the study include its open-label design and the concomitant administration of high-dose glucocorticoids in all patients and the use of plasma exchange in some patients. Nonetheless, these are powerful observations that represent the foundation for an ongoing, large multi-center controlled trial of Rituximab in ANCA-associated vasculitis sponsored by the Immune Tolerance Network. Of note, similarly promising results have also been independently reported in a second series of 9 patients with ANCA-associated vasculitis (143).

6. CHARACTERISTICS OF B-CELL DEPLETION AND RECONSTITUTION

By and large, Rituximab has been remarkably effective for the depletion of non-malignant B cells in human subjects treated either for lymphoma or autoimmunity (114, 144). It is important to bear in mind however that in many cases, Rituximab was administered in combination with other cytotoxic drugs. It is also worth mentioning that studies thus far have concentrated on the analysis of peripheral blood and that systematic studies of bone marrow and peripheral lymphoid tissue (secondary or tertiary) are still lacking. Such studies will be of the utmost importance given the clinical and immunological implications of different types of B-cell depletion (discussed below in more detail) and current evidence indicating the differential susceptibility of specific B-cell compartments and the influence exercised by the microenvironment (145, 146). Thus, when mice expressing transgenic human CD20 were treated with Rituximab, both germinal center and resident marginal zone B cells were relatively resistant to elimination. Nonetheless, marginal zone B cells were rendered susceptible to Rituximab by being mobilized into the intravascular space (145). On the other hand, elimination of >95-98% of mouse bone marrow, spleen, lymph node and gut-associated B cells has been reported using mouse anti-CD20 IgG2a antibodies suggesting that more universal depletion of tissue-bound B-cells may be achievable by enhancing the avidity of therapeutic antibodies for specific FcγR (in particular human FcγRIIIa)(146).

Collectively, less than 10% of patients reported in the literature that received Rituximab for different autoimmune diseases failed to deplete PBL B-cells (in most cases depletion has been defined as achieving levels of < 5 $CD19^+$ B-cells/μl) (108, 110, 116-118, 120, 122, 123, 147, 148). Our own studies in SLE, as previously discussed, found a higher rate of non-depleters, at least in part due to the use of low-doses of Rituximab in some patients and the low-intensity of concomitant cytotoxic therapy, which may have also facilitated the production of higher levels of HACA antibodies (10, 132). In these patients, residual B cells were heterogeneous and included cells with the following surface phenotypes: Bm1/2 (naïve); Bm2' (presumably including pre-germinal center and transitional cells); Bm5 (memory); and plasma cells. On the other hand, two main subsets account for the vast majority of residual cells in patients that experienced good depletion: $CD20^+$ isotype-switched memory cells and to a lesser degree, $CD20^-$ plasma cells (131). Yet, it should be noted that in these patients a great majority of pre-treatment plasma cells are eliminated within the first two months after treatment, suggesting that the life-span of recirculating plasmablasts typically expanded in patients with active SLE is relatively short. The predominance of isotype-switched memory cells observed among residual B-cells could be explained by incomplete interruption of germinal center reactions. However, this possibility remains to be formally addressed by analysis of secondary lymphoid tissue, currently underway in our laboratory.

Most studies have found that maximal depletion is achieved 1-2 months after treatment. In general reconstitution appears to start (as defined by total numbers of B-cells raising above 5/μl) from 3-8 months post-Rituximab although some patients remain depleted for up to several years (130, 149, 150). On the other hand, once B cells reemerge, the kinetics of repopulation appears to vary greatly. This situation is illustrated by an analysis of RA patients recently published. These patients segregated into two groups, one in which normalization of B-cell numbers occurred quickly after the initial reappearance of B cells (4-8 weeks) and a second group in which this process took several months (148). Interestingly, in both groups B-cell depletion was associated with increased serum levels of BlyS (B lymphocyte stimulator) and decreased BlyS levels preceded subsequent B cell expansion. Whether this counterintuitive inverse relationship between serum BlyS and B-cell numbers reflect the amount of BlyS soaked up by B cells remains to be determined. In contrast, our data indicate that the main feature distinguishing SLE patients is the quality and kinetics of B-cell repopulation rather than the timing of B-cell reappearance. The implications of these differences for disease outcome and immunological tolerance are discussed below in detail.

7. IMMUNOLOGICAL CONSEQUENCES OF B-CELL DEPLETION

It is apparent that B-cell depletion may impact both the physiological and pathological functions of B-cells. One obvious way of assessing such impact is through the analysis of serum antibody levels, with the caveat that plasma cells are largely responsible for antibody production. Given that mature long-lived plasma cells lack expression of CD20 and are consequently impervious to treatment with Rituximab, it is not surprising that the collective observation has been that total antibody levels remain stable for long periods of time after B-cell depletion. Similarly, pre-existing anti-tetanus toxoid and anti-pneumococcal polysaccharide antibodies remain stable for prolonged periods of time (10, 123, 148). This preservation of pre-existing humoral memory may be in part responsible for the safety profile of Rituximab. However, several caveats are worth bearing in mind. Hypogammaglobulinemia with low IgG levels has been observed in children treated with Rituximab and IVIG has been advocated in this group as well as prophylactically in infants (119, 151, 152). In addition, the ability of B-cell

depleted patients to respond to immunization either with neoantigens or recall antigens is significantly impaired (129, 153, 154).

The effect of Rituximab on disease-associated autoantibodies has been more difficult to understand due to heterogeneity among patients, differences between different diseases and the use of other immunosupressive modalities in most studies. Still, significant drops in autoantibody levels have generally been observed in most diseases. Thus, a significant decline in RF levels has been reported in RA patients despite stable levels of total and anti-microbial antibodies and a substantial decline or disappearance of ANCA levels and anti-platelet antibodies has been reported in patients with ANCA-associated vasculitides and autoimmune thrombocytopenia respectively (1, 120, 142, 148). Substantial decreases in autoantibodies have also been observed in patients with IgM antibody-associated polyneuropathy. In the latter studies however, total IgM levels declined to a similar extent as IgM autoantibodies (115).

The disproportionate decline of some autoantibodies as compared to total immunoglobulins is consistent with the notion that steady-state serological memory is largely maintained by long-lived plasma cells (155, 156). By the same token, the decline of autoantibodies in this context seems to imply that their production is differentially regulated and may be dependent upon the continuous generation of short-lived plasma cells from follicular or extra-follicular autoreactive B-cell precursors that are effectively targeted by Rituximab (157-163). Along these lines, it will be important to determine the efficacy of Rituximab in depleting ectopic lymphoid aggregates (with or without germinal center-like structures) that are likely to be a major source of autoantibodies, at least in some diseases (121, 164, 165).

In at least some diseases (such as RA, ANCA-associated vasculitis, IgM antibody-associated polyneuropathy and inhibitory anti-Factor VIII antibodies in patients with Hemophilia), a decline in autoantibody titers seems to be associated with clinical improvement (142, 148, 166). This correlation however has not been observed in patients with ITP (1). Subsequent rises in autoantibody titers (RF, ANCA) seem to be closely associated with B-cell repopulation and sometimes may even precede the expansion of B-cells (148). Interestingly, the reemergence of serum IgM RF appears to be closely associated with disease relapse (149). This is also likely to be the case for ANCA antibodies, although in most instances prophylactic re-treatment was initiated following a rise in ANCA titers, thereby precluding a clear assessment of the likelihood of clinical relapse (142). Collectively, the data suggest that at least in some patients repopulation may be dominated by the preferential expansion of residual autoreactive B-cells, perhaps due to a competitive advantage (or lack of competition) in a depleted peripheral compartment (131, 149, 167-169).

As shown by our studies, the picture is more complicated in SLE where total IgM and IgG antibody levels as well as disease-specific autoantibodies (both anti-dsDNA and 9G4 antibodies) remained stable for at least 1 year. Strikingly, such persistence occurred despite profound B-cell depletion, early clinical improvement and elimination of autoreactive memory B-cells (as measured by $9G4^+$ B-cells). Upon longer follow-up however, SLE patients with good B-cell depletion could be split almost evenly into those whose anti-ds DNA levels progressively declined after 1 year and eventually normalized and those in whom the levels failed to diminish substantially (10, 131). As a group, serological responders experienced a resolution of the B-cell abnormalities present at baseline and included the patients with more dramatic and sustained clinical responses (see below). These observations suggest that SLE anti-dsDNA and 9G4 autoantibodies are produced to a large extent by long-lived plasma cells. In addition, it seems likely that tissue depletion of autoreactive B cells that may continue to generate plasma cells of heterogeneous life span may have been less successful in patients with good blood depletion but poor serological response.

8. CAN IMMUNOLOGICAL TOLERANCE BE RESTORED BY B-CELL DEPLETION?

B-cell depletion has the potential to induce disease amelioration by inhibiting autoantibody production or by interfering with other B-cell pathogenic functions previously discussed in this review. However, such improvement will be necessarily transient unless the absence of B cells can successfully restore immunological tolerance. Admittedly, the definition of tolerance is arbitrary and its assessment in humans is a tall order. Nonetheless, thus far the norm is that disease relapse ensues after B-cell repopulation although the interval between relapse and B-cell reconstitution is highly variable between diseases and individual patients (130, 136, 142, 149). This general rule applies to patients with SLE, RA and WG and clearly indicates that tolerance has not been fundamentally restored. However, in all these diseases there appears to exist a subset of patients (15-35%) who remain in remission despite the reemergence of B cells. While in RA and WG follow-up periods thus far reported are relatively short (less than 15 months), this observation raises the tantalizing possibility that tolerance may have been restored in such patients and begs the question as to what distinguishes this subset.

Our studies in SLE patients offer initial insight into this critical question. Thus far, we have carefully studied 17 patients included in our original trial for up to 5 years after treatment with Rituximab (10, 131, 150, 170). On the basis of these studies, our patients can be classified into 3 different groups:

A) Patients who achieve profound B-cell depletion and in whom serum anti-DNA antibodies and 9G4 antibodies normalize after an average of 16 months post-treatment. Strikingly, some of these cases became and have remained ANA negative. These patients experienced complete clinical remission which has been maintained with no significant immuno-suppression for up to 3-5 years after treatment.

B) Patients with good initial B-cell depletion but no decline in autoantibodies and transient clinical response.

C) Patients with incomplete B-cell depletion and no significant clinical improvement.

Interestingly, patients in group A are characterized by an over-correction of the B-cell depletion in such a way that after approximately 1 year the peripheral blood B cells represent 20-45% of all mononuclear cells. Strikingly, up to 80% of all PBL B cells in these patients display a transitional phenotype and the memory B-cell compartment remains significantly shrunk for up to 3-5 years after treatment (representing only 6.3 ± 0.9% versus 30.5 ± 6.9 % in normals, p=0.0000046). In contrast, patients in group B are characterized by a predominance of memory B-cells during their recovery and for up to 3-5 years of follow-up (Figures 1 and 2). Interestingly, a similar predominance of transitional B cells and scarcity of memory B cells has been reported during the writing of this review in RA patients although after considerably shorter follow-up. In this case however, although memory B cells tended to be lower in patients with sustained clinical remission, the differences between groups did not reach statistical significance (149).

In essence, the repertoire of SLE patients from group A, closely resembles the neonatal B-cell compartment with the meaningful difference that the accumulation of memory B cells is even slower than during original ontogeny (171). These observations are consistent with the notion that these patients are undergoing a slow *de novo* immune reconstitution without reemergence of autoimmunity as indicated by the disappearance of autoantibodies (despite the maintenance of normal serum total immunoglobulin levels) and by the normalization of the levels of autoreactive 9G4 B-cells which are typically expanded within the memory compartment of patients with active SLE (172, 173) (Figure 3). In contrast, the clinical and immunological profile of patients in group B raises the possibility that the reconstitution of their B cell compartment may be driven by the expansion in a lymphopenic environment of residual memory B cells enriched for autoreactivity.

If indeed, immunological tolerance has been re-established in these patients, what mechanisms could account for this phenomenon? Essentially, two non-mutually exclusive models can be proposed:

Tolerance model: B-cell depletion and the ensuing B-cell reconstitution provides a second chance for the establishment of B-cell tolerance. This model is based on the assumption that tolerance is broken by the impact on genetically susceptible B cells of environmental and/or stochastic events during the generation and maturation of the B-cell repertoire (174). This model postulates that upon reconstitution, the convergence of these factors that were originally responsible for the breakdown of B-cell tolerance will not recur and previously defective tolerance checkpoints will now be operative in censoring autoreactive B cells.

Regulatory model: As previously discussed, B cells play multiple immuno-modulatory roles. The absence of B cells could have the following beneficial consequences:

Inhibition or attenuation of priming, clonal expansion and activation of autoreactive T cells. Furthermore, it can be postulated that upon reconstitution, predominantly transitional/naïve B-cells devoid of co-stimulatory ability could contribute to the induction of T cell tolerance (175-177). Interestingly, SLE patients treated with Rituxan who experienced a fast clinical response had significantly lower frequencies of $CD4^+$ $CCR7^-$ effector memory T cells (178). This work has only been presented in abstract form at the 2004 ACR meeting. In the only published work thus far looking at T cells in SLE after Rituxan therapy, the numbers of $CD40L^+$ $CD4^+$ T cells were significantly decreased as early as 1 month post-therapy (in the presence of profound B-cell depletion) (179). Interestingly, these numbers continued to be reduced over time and correlated with the attainment and maintenance of clinical remission. Of note, significant amounts of steroids (0.5 mg/kg/day) were used for several weeks and could have had a significant impact on the results (180).

Promotion of Th1 differentiation, since in the absence of B cells the over-representation of IL-12 producing dendritic cells among APCs should strongly favor a Th1 shift. It has been postulated, however, that Rituximab-induced down-regulation of CD80/86 on B-cells might induce a Th2 shift in SLE (139). Whether this is indeed the case and it correlates with a favorable clinical response remains to be determined, as the view of SLE as a purely Th2 disease is rather simplistic (139, 181-183). The effect of B-cell depletion on Th1/Th2 balance has not yet been formally studied.

Expansion of T regulatory cells (76, 77).

Elimination of IFNα-inducing immune complexes, whether by decreased levels of the corresponding autoantibodies or by lower levels of available apoptotic/necrotic cell targets. Interestingly in this regard is our observation that SLE patients in group A are characterized by the absence of anti-RNP autoantibodies, perhaps the most potent autoantibody inducers of IFNα (168, 170, 184).

Of note, we believe that mechanisms from both models need to be invoked in order to explain our observations in Group A of Rituximab-treated patients. Indeed, similar profiles of reconstitution with transitional cells predominance and delayed memory re-accumulation have been reported not only in neonates but also after bone marrow transplantation (185). However, a critical difference merits consideration if we are to understand the implications of this observation. Thus, the slow maturation of the B-cell compartment in both neonates and post-bone marrow transplants is likely to be explained at least in part by immaturity or disruption of secondary lymphoid tissue and by slow maturation or decimation of the T cell repertoire, respectively. Therefore, it is tempting to speculate that similar changes in lymphoid development or

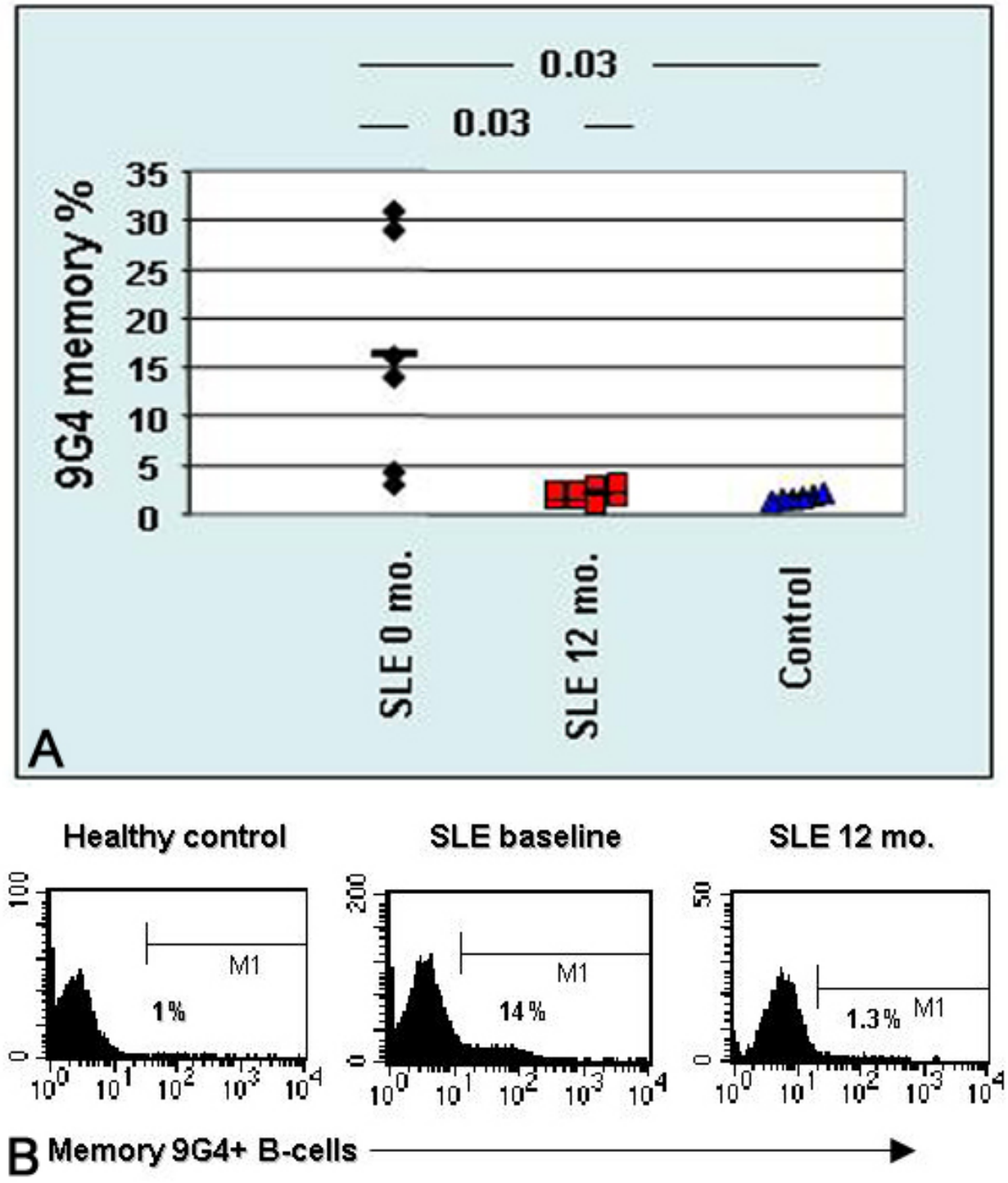

Figure 1. B cell reconstitution after B cell depletion in SLE. Flow cytometry analysis of peripheral blood B cells in a representative SLE patient from Group A. Staining of CD19+ B cells with anti-CD27 and anti-IgD antibodies reveals a substantial decrease post-treatment of both switched (sM) and non-switched (nsM) memory B cells as well as CD27+++ plasmablasts (PB) as compared to baseline. Strikingly, the scarcity of memory cells and dominance of cells with a presumed naïve (N) phenotype are sustained even after 3 years post-treatment. Closer analysis of a similar SLE patient post-Rituximab indicates that in addition to naïve B-cells, the CD27-negative population includes a population of CD24high CD38high transitional B cells (T) which is significantly expanded as compared to healthy adult controls with levels similar to those found in cord blood.

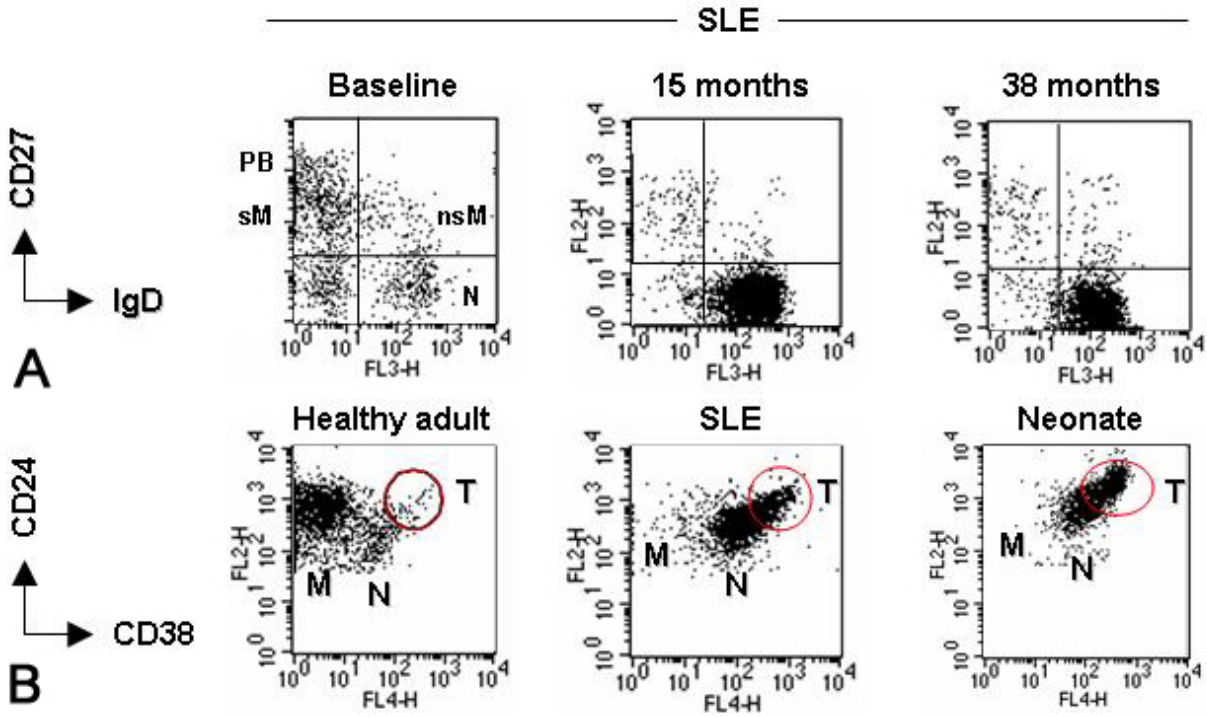

Figure 2. Delayed B cell memory reconstitution after B cell depletion in SLE. SLE patients treated with Rituximab were analyzed 3-5 years after treatment for their frequency of IgG+ CD27+ memory B cells using flow cytometry. The relative frequency of these cells amongst all CD19+ peripheral blood B cell is shown as compared to normal values. Patients experiencing sustained clinical remission show much lower levels than SLE patients in other response groups and normal controls.

microarchitecture and in T cell activation or survival may have been triggered by the prolonged absence of B cells in SLE patients in group A (Figure 4) This possibility is all the more tantalizing when one considers that SLE patients should have a pre-formed, activated and presumably expanded autoreactive T cell memory that could be sustained by ubiquitous self-antigens and that autoreactive T cells have the potential to break tolerance in the reemerging B cell compartment (186, 187). Accordingly, we would hypothesize that B cell depletion must trigger fundamental changes in the quality of the newly developed B-cell compartment and/or in the pre-treatment autoreactive T cell compartment. In turn, the consequences of B-cell depletion (distortion of lymphoid architecture, diminished T cell help, reduction in DC function, decreased BLyS) may delay the maturation of a new B-cell compartment. Similarly, the recent demonstration that Tregs may directly suppress B cells suggests that if Tregs are expanded upon B-cell depletion this expansion could in turn contribute to downregulate newly emerging B cells (188, 189).

9. PERSPECTIVES IN B-CELL DEPLETION. CHALLENGES AND OPPORTUNITIES

The immediate future of therapeutic B-cell depletion will certainly include the performance of multi-center, controlled trials (many of them currently underway or in preparation) to formally establish the efficacy of this intervention in multiple autoimmune diseases. Moreover, investigators undoubtedly will determine the relative efficacy of B-cell depletion alone or in combination with other forms of immuno-suppression. Of particular interest will be to perform studies of B-cell depletion with combinations of different biological agents capable of either killing or modulating B-cell function or survival as well as agents with the ability to target plasma cells (1, 105, 108, 110, 190-192). Particularly intriguing combinations include antibodies against other B-cell markers including those expressed at earlier developmental stages than CD20 (193), B-cell inhibitory receptors such as CD22 or FcγRIIb, BLyS antagonists, anti-type I interferon agents and oligonucleotides with the ability to modulate specific B-cell subsets or other agents capable of inhibiting TLR co-stimulation (190, 194-197). Other foreseeable refinements in B-cell depletion include the utilization of fully human anti-CD20 antibodies that elicit a lesser immunogenic response from the host and can be safely used to treat patients that develop HACA responses to Rituximab (198).

We will also need to learn the place of B-cell depletion in the therapeutic hierarchy, including its efficacy for induction of remission versus maintenance of remission and its potential for the rescue of disease refractory to other

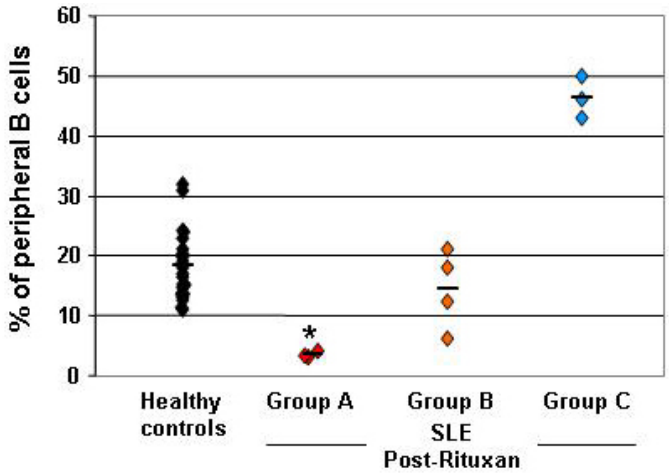

Figure 3. Normalization of autoreactive B-cells in SLE patients after B cell depletion. The frequency of autoreactive 9G4+ CD27+ memory B-cells measured by flow cytometric analysis of peripheral blood cells is shown for a cohort of SLE patients at baseline and after B cell reconstitution following treatment with Rituximab. The increase in 9G4+ memory cells typically observed in active SLE patients was corrected after B cell reconstitution to levels similar to those observed in normal controls (Adapted with permission from 131). Flow cytometry analysis of peripheral blood B cells from a representative SLE patient is shown at baseline and one year after treatment in comparison to a healthy control.

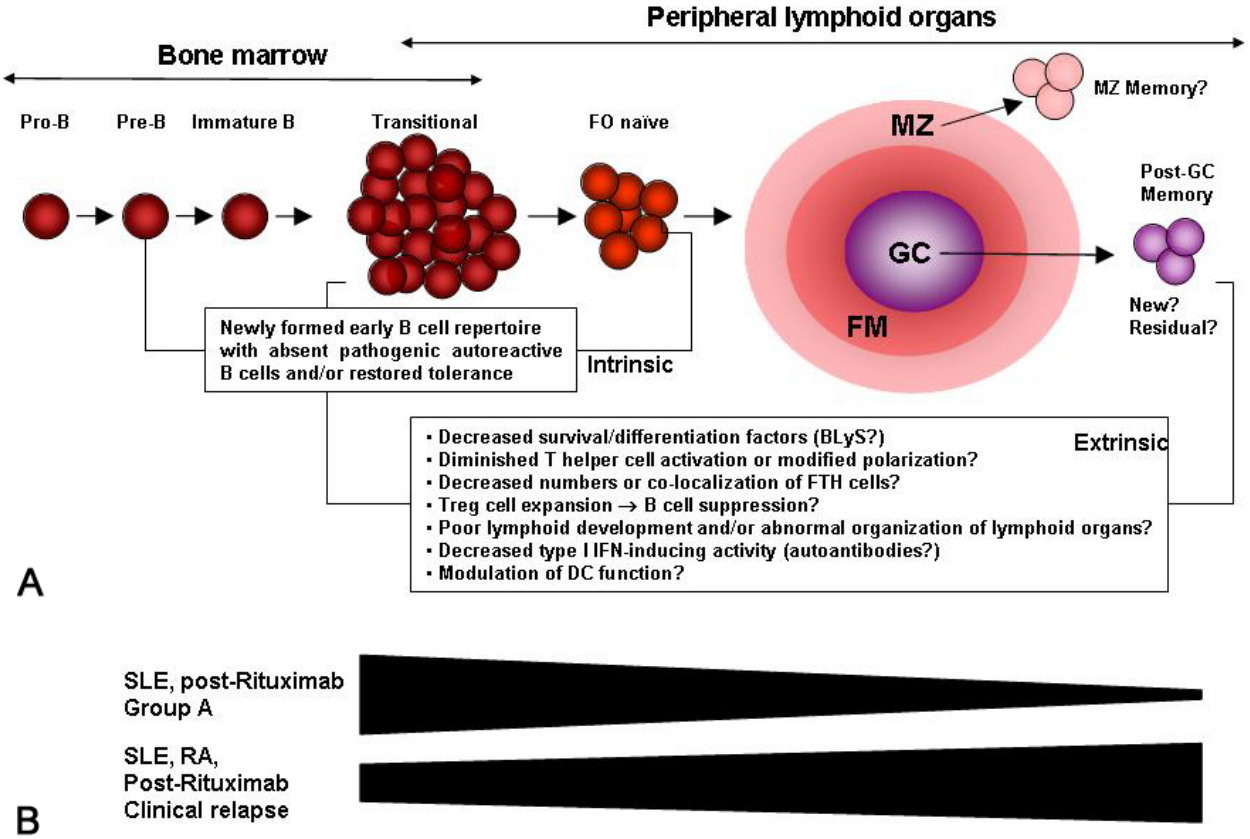

Figure 4. Theoretical model to account for B cell reconstitution after Rituximab. SLE patients experiencing prolonged remission after B cell depletion display a predominance of transitional and naïve B cells and decreased B cell memory compartment in contrast with patients with either RA or SLE that experience a disease flare after treatment with Rituximab. The phenotype of the former subset of patients could be explained either by a change in the intrinsic properties of the emerging B cells, by extrinsic factors that modulate B cell differentiation, selection, activation, survival and tolerance or most likely by a combination of both. Whether the memory B cell expansion in the latter group represents residual autoreactive cells and whether autoreactive B cells are differentially regulated in different compartments remain to be determined. FO: Follicular; FM: follicular mantle; GC: germinal center; MZ: marginal zone

modalities. Two areas of particular importance are the utilization of B-cell depletion to treat early disease instead of advanced or refractory disease and the safety and efficacy of repeated cycles of treatment to treat and even prevent disease relapses frequently associated with B-cell repopulation. While the currently available information is encouraging on all counts regarding retreatment, larger studies with longer periods of observation will be required (142, 148, 199). Meanwhile, a measure of caution should be exercised on the basis of isolated reports of severe hepatitis B in patients treated with Rituximab (200-202). Whether these cases reflect a role for B cells in the control of Hepatitis B infection remains to be established (81). We will probably need to establish the need for pre-treatment screening and risk stratification for some infectious diseases similar to the tuberculosis screening currently performed prior to anti-TNF therapy. Furthermore, the long delay observed in the reconstitution of B-cell memory dictates the need for new studies to understand how pre-established memory may be lost in these patients and how to develop effective protocols for immunization.

Several additional challenges and opportunities will arise from the expanding utilization of B-cell depletion. Thus, ample opportunity should be available for the astute investigator to understand the differential role of B-cells in different diseases, the impact of B-cell depletion on other arms of the innate and adaptive immune system and the relative contribution of these mechanisms to disease amelioration. Finally and perhaps most challenging, we will have a unique opportunity to elucidate whether and how immunological tolerance can be restored.

An especially interesting area for future studies will be to understand the role of B cells and B-cell depletion in other diseases including multiple sclerosis and Type 1 diabetes, scleroderma and liver disease (8, 36, 103, 203). Other medical areas where B-cell depletion could find important applications include humoral organ rejection (where antibodies could play different roles) (204, 205), chronic obstructive pulmonary disease and asthma (where B-cells can be prominently found in the parenchyma and airways respectively) (206) (207) and even in cancer therapy (given the observation that B-cells may inhibit CTL-mediated tumor immunity and the absence of B cells may enhance the efficacy of tumor vaccines) (208, 209).

Due to the anticipated widespread use of B-cell depletion in the future, perhaps it would be wise to end this review considering other possible undesirable effects of this intervention. It is obvious that in addition to adaptive memory responses previously discussed, B cells play important protective roles in autoimmunity and infections including the generation of natural autoantibodies, the response to vaccination with pneumococcal capsular polysaccharides, viral immunity, clearance of apoptotic cells, protection against atherosclerosis and the suppression of pathogenic autoantibody responses (55, 81-85, 210-217). Moreover, as previously discussed, B cells may play a protective role by suppressing autoimmune responses by multiple mechanisms and may promote Th2 differentiation. Therefore, it is plausible that prolonged absence of regulatory mechanisms provided by B cells or undesirable Th1 deviation might have adverse effects, with exacerbation of autoimmunity in some cases (218, 219). Consequently appropriate vigilance will have to be observed in order to better understand the potential risks of intense, chronic B-cell depletion.

10. ACKNOWLEDGEMENTS

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