antibodies

Review

Role of B Cells in Breaking and Maintaining Tolerance to Clotting Factor VIII in Congenital and Acquired Hemophilia A

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Abstract: Immune responses directed against clotting factor FVIII (FVIII) seriously complicate treatments for patients with hemophilia A. This response can manifest in congenital hemophilia A patients who generate inhibitor antibodies that bind and inactivate "transplanted" replacement FVIII, as well as in acquired hemophiliacs, whose immune systems have lost tolerance to self-FVIII. Regardless of the mechanism by which production of anti-FVIII inhibitor antibody is triggered, the maintenance of this deleterious response in both congenital and acquired hemophiliacs likely relies upon FVIII specific memory B cells. In this review, the similarities and differences in the kinetics, specificities, and subclasses of antibodies produced in response to allo- and auto-FVIII is outlined. A brief description of the immune cell interactions that contribute to maintenance of antibody response, focusing on development of memory B cells and/or long lived plasma cells is also presented. As current treatments for inhibitor antibodies are not successful in all patients, a better understanding of the functions and persistence of memory B cells specific for FVIII is required. Herein, both clinical and experimental data regarding the effects of immune tolerance induction on memory B cell subpopulations is discussed. Finally, the outcomes of B cell-specific depletion via rituximab in hemophilia and other autoimmune diseases are discussed to highlight insights into the subpopulations of memory B cells that contribute to the development and maintenance of successful tolerance to FVIII.

1. Introduction

While acquired immunity is necessary for human heath, autoimmune response against self antigens represents a significant medical problem. In the case of hemophilia, intriguing comparisons can be made between patients with congenital hemophilia A, who generate an acquired immune response against "transplanted" replacement FVIII, and acquired hemophiliacs, whose immune systems have lost central tolerance to self FVIII antigen. The outcome in both instances is the production of functionally inhibitory anti-FVIII antibody which complicates treatment of the disease. The development and maintenance of the B cell response in these patient populations may represent vastly different responses to the same antigen.

2. Congenital Hemophilia A *vs.* Acquired Hemophilia

A mutation or a deletion in the region of the X chromosome that encodes clotting factor VIII (FVIII) results in congenital, or inherited, hemophilia A (HA), which has a prevalence of 1–5,000 male births. Patients afflicted with severe hemophilia (<1% FVIII activity) suffer from spontaneous muscle and joint bleeding leading to the crippling effects of hemophilic arthropathy. Without FVIII treatment, the life expectancy for a severe hemophiliac is ~20 years. Unfortunately, treatment with therapeutic FVIII often results in "non-self" recognition of this protein by the immune system, culminating in the formation of pathogenic anti-FVIII inhibitor antibodies (Abs) capable of neutralizing FVIII in approximately 30% of patients. Subsequent bleeding episodes are difficult to manage and result in significant mortality and morbidity, a reduction in quality of life, and a significant financial burden averaging nearly $900,000/patient/year [1].

Acquired hemophilia is the manifestation of an autoimmune response generated against self-FVIII. Despite its low rate of occurrence (prevalence of 1:1.5 million/year), it is a remarkably damaging disease, with reported mortality between 6%–22% and an estimated cost of ~$30,000/resolved bleed/patient. In contrast to congenital HA, which affects pediatric patients, inhibitor antibody in acquired hemophilia is associated with an aged patient population. In approximately 50% of patients with acquired hemophilia a specific co-morbidity (autoimmune disease, malignancy, pregnancy) can be identified. The remainder develops spontaneous, idiopathic inhibitors.

3. FVIII Structure, Function, and Inhibition

FVIII, a co-factor for activated FIX in the intrinsic coagulation pathway, can be synthesized in multiple cell types, including PBMC [2], but is secreted primarily by sinusoidal endothelial cells of the liver as a heterodimer. It consists of a ~200 kDa heavy chain (comprised of the A1, A2, and B domains) and an 80 kDa light chain (made up of the A3, C1, and C2 domains). Epitope mapping of anti-FVIII antibody specificity has been performed for Abs isolated from congenital HA patients as well as acquired hemophilia patients. In both patient populations, the majority of inhibitor Abs bind to the A2, A3, and C2 domains. Anti-C2 Abs inhibit binding to phospholipid and VWF, shortening the

half-life of FVIII in circulation. Anti- A2 and A3 domain antibodies interfere with FIXa and FX binding, which prevents the assembly of the factor Xase complex, resulting in inadequate generation of thrombin [3–11].

4. Characteristics of Allo- and Auto-Antibody Response to FVIII

Allo-immune response to FVIII is most likely to develop in pediatric patients within 12–20 FVIII treatments [12]. The majority of patients with severe inhibitors develop a polyclonal response consisting of primarily IgG1 and IgG4 subclasses of antibody that inactivate FVIII via a type I pattern with linear, second order kinetics [10]. A recent study using sensitive surface plasmon resonance (SPR) to investigate subclass specific binding to FVIII demonstrated that early anti-FVIII immune response in AH patients is characterized by primarily IgG1 while the IgG4 subtype is produced later, suggesting the gradual development of a polyclonal response caused by repeated doses of antigen over time [13]. Interestingly, epitope specificity does not appear to correlate with specific isotypes, rather, it has been suggested that IgG class switching occurs after epitope specificity is determined [13,14].

The kinetics of the development of the autoimmune antibody response to FVIII are less well understood, as studies of FVIII specific inhibitors in patients can only be undertaken once the hemophilia phenotype is presented. In contrast to binding kinetics observed for FVIII inhibitors in HA patients, autoimmune inhibitors have been found to rapidly neutralize FVIII procoagulant activity following non-linear linear type II kinetics [15]. This physiolology appears to affect the manifestation of the disease—in contrast to hemarthrosis commonly observed in congenital hemophilia A patients with inhibitors, patients with acquired hemophilia usually present with soft tissue and mucosal bleeds. Despite the difference in pathological outcome, anti-FVIII auto-antibodies isolated from patients with acquired hemophilia appear to be remarkably similar to allo-antibodies directed against FVIII. The polyclonal response is also characterized by IgG1 and IgG4 subclasses, and these share the same epitopes as anti FVIII allo-antibodies [13,16,17]. It has been suggested that the differences in FVIII binding kinetics and the manifestation of bleeding might be related to a preponderance of antibody specific for one FVIII epitope, rather than the mix of many epitopes observed in patients with congenital hemophilia that develop inhibitors [13,16]. It is also possible that a much larger plasma cell population exists in patients with acquired hemophilia. Indeed, it has been demonstrated in models of other autoimmune disorders that a large number of antibody secreting cells can develop very rapidly [18–22].

5. Anti-FVIII Antibody Response—A Break in Tolerance

B cell differentiation into antibody-secreting plasma cells is dependent upon their capacity to activate in response to recognition of foreign antigens while avoiding reactivity to self. This relies on both central and peripheral tolerance (for more in-depth descriptions of these concepts, the reader is directed to recent excellent reviews [23–25]). As mutations in the F8 gene result in production of incomplete FVIII protein it is reasonable to assume that FVIII-reactive B cells escape deletion or receptor-editing in the bone marrow of congenital hemophilia patients, a failure of central tolerance. Indeed, the severity of the F8 gene mutation has the greatest influence on a patient's risk for developing inhibitors. Patients with large deletions, nonsense mutations, and chromosomal inversions have the highest incidence of severe inhibitor formation [26]. Further experimental evidence for the

necessity of FVIII expression during lymphocyte development was provided by gene therapy studies in animal models that showed that stable expression of FVIII protein in thymus or bone marrow correlated with tolerance to FVIII [27,28]. Thus, it is likely that an existing population of FVIII-reactive B and T cells exists in congenital hemophilia patients, and these cells recognize injected therapeutic FVIII as "non-self". In contrast, the development of the antibody response in acquired hemophilia likely represents a failure to maintain peripheral tolerance to endogenous FVIII in the mature lymphocyte population. Lymphocytes reactive to self FVIII survive in the periphery and are not maintained in an anergic state due to a breakdown in immune suppressive mechanisms.

6. Development of Inhibitor Antibodies

Development of inhibitor antibody in congenital hemophilia patients receiving therapy most likely occurs via generation of an adaptive T cell dependent immune response. Recombinant FVIII is injected, circulates through the blood, and is taken up into the lymphatic tissue of the spleen. In a FVIII-deficient HA mouse model, radiolabeled human FVIII has been detected in spleen as early as 10 minutes post-injection where it co-localizes with antigen-presenting cells (APC) in the marginal zone (MZ) within 30 minutes [29]. APC presentation of FVIII together with the up-regulation of co-stimulatory molecules then activates CD4+ T helper cells that, in turn, migrate to the edge of the splenic follicle to provide help to B cells. Differentiation into plasma cells then occurs in the germinal center, and FVIII specific antibody is secreted. The importance of CD4+ T cell help in this process has been demonstrated by the finding that blockade of CD4+ T cell co-stimulatory molecules such as ICOS and CD40 ligand (CD40L) reduces inhibitor antibody and allowed the restoration of FVIII activity [30–35]. Clinical evidence supporting a necessary role for T cell help was provided by the finding that low CD4 cell counts in Human Immunodeficiency Virus infected hemophiliacs correlated with the disappearance of FVIII inhibitor antibodies [36].

In acquired hemophilia, the pathway to production of inhibitor antibody is less clear. Studies of antibodies isolated from humans, as well as from humanized SCID mice, have demonstrated that anti-FVIII IgG antibody is produced at low levels in healthy subjects [37,38]. However, this antibody is not inhibitory, perhaps because of the presence of regulatory anti-idiotype Abs in normal conditions (reviewed in [39]). Additionally, CD4+ T cells isolated from healthy human subjects are capable of short-term proliferation when cultured with FVIII [40]. Hence, a break in the mechanisms of peripheral tolerance and stimulation of pre-existing B cell populations producing anti-FVIII Abs may trigger the autoimmune response to FVIII. In acquired inhibitor patients who are also afflicted with autoimmune disorders, such as lupus, the T and B cell activation that occurs during the response to nuclear antigens may also stimulate pre-existing FVIII-specific B and T cell activation. Similarly, alterations in B cell signaling in blood cancers might also allow for the survival B cell clones reactive to self-FVIII [41]. However, these explanations do not account for the mechanisms that drive idiopathic acquired hemophilia production. Regardless of the trigger, the development of plasma cells secreting FVIII-specific autoantibody is likely to share characteristics with other autoimmune disorders. Studies in mouse models of lupus and rheumatoid arthritis have demonstrated that autoantibody may be secreted by short-lived plasmablasts that develop independent of germinal centers [18,19,42] Extrafollicular T-independent, hypermutated autoantibody responses may also

develop via TLR stimulation [20–22]. Therefore, it is likely that the plasma cells that secrete autoantibody directed against FVIII in acquired hemophilia patients utilize very different signaling pathways and this may account for some of the differences in antibody kinetics as described earlier.

7. Role of Memory B Cells in Inhibitor Production

Inhibitor antibodies are secreted by plasma cells found in spleen and bone marrow [43], however, it is unknown if these cells are an example of "long-lived" plasma cells maintained in bone marrow or if they are steadily generated from a pool of FVIII-specific memory B cells, which differentiate into short-lived plasma cells [44]. Evidence for the former was provided by a study in the mouse identifying plasma cells secreting anti-FVIII antibody at 22 weeks post FVIII immunization [43]. On the other hand, low level chronic antigen exposure appears to encourage development of short-lived plasma cells in spleen [45]. Most HA patients on prophylactic therapy receive continuous low dose FVIII injections (25–40 IU/kg injections 3×/week), therefore, this might favor development of memory B cells that can transition to short-lived plasma cells in the spleen following exposure to FVIII. Indeed, it has been determined that 0.07%–0.35% of circulating memory B cells isolated from peripheral blood of HA patients are capable of differentiating into plasma cells upon re-exposure to FVIII [46,47]. Multiple reports have demonstrated a critical role for CD4+ T cell help in this process [30–36,47]. On the other hand, recent experimental evidence suggests that T-independent activation of memory B cells specific for FVIII can occur by co-stimulation of TLR 9 or TLR 7 in the presence of dendritic cells [48,49]. As MZ B cells can respond to antigen via both T-dependent and T-independent mechanisms (reviewed in [50]), it is intriguing to speculate that this population may play an important role in maintenance of inhibitor antibody response in both congenital hemophilia patients and acquired hemophiliacs.

The role of memory B cells in the maintenance of pathogenic antibody production in acquired hemophilia patients is currently unknown. However, there is both experimental and clinical evidence that long-lived memory B cells do indeed develop in autoimmunity [51–53]. These cells appear to share the defining characteristics of memory B cells, in that they require T cell help to form, remain in a resting state in the absence of antigen, and can rapidly differentiate into antibody secreting cells upon re-challenge [51]. If such a population of FVIII-specific memory B cells exists in acquired hemophilia, it might be responsible for the relapses following treatment observed in some patients.

8. Clinical Treatments for Inhibitor Antibodies

The most effective method to ablate high titer inhibitor antibodies in congenital HA patients is immune tolerance induction (ITI). Though the use of one of several protocols in use worldwide, tolerance to injected FVIII can be achieved by regular administration of FVIII (with or without concomitant immunomodulation) over a period of months to greater than two years [54–57]. ITI has been demonstrated to be successful in abating functional inhibitor antibodies, normalizing the half-life of injected FVIII, and preventing bleeding episodes in approximately 80% of patients [56,57].

Due to the rapid rate of antibody binding to and inactivation of FVIII in acquired hemophilia patients, ITI via high dose FVIII injection is usually ineffective. Instead, inhibitors are managed by immunomodulatory therapies. The most commonly used are corticosteroids, used either as a single

agent or in combination with azathioprine or cyclophosphamide. As relapse occurs in 10%–20% of acquired hemophilia patients (ranging from one week to 11 months post-treatment), other immunomodulatory agents have been added to steroid therapy in an attempt to improve outcome [58]. These agents include intravenous immunoglobulin, cyclosporine, and rituximab. Given the low incidence of acquired hemophilia and associated co-morbidities, large scale clinical studies regarding efficacy of these treatments have yet to be completed.

9. Effect of Tolerance Induction on Memory B Cells

FVIII specific memory B cells were not detectable in the majority of HA patients successfully treated with ITI [46,59], suggesting that tolerance to FVIII results in depletion of this subset. In support of this, data generated in the mouse model indicated that high concentrations of FVIII inhibit FVIII-specific memory B cell differentiation into Ab-secreting plasma cells in culture, and this may be due in part to induction of apoptosis [4]. Additionally, plasma from congenital HA patients that undergo successful ITI contains anti-idiotypic antibodies that bind to FVIII recognition sites on inhibitor antibodies. It is conceivable that these newly formed Abs might interact with the inhibitory FcγRIIB to prevent B cell activation [12]. Finally, some evidence suggests that tolerance induction restores a "normal" balance of memory B cells. A recent report determined that the total population of CD19+CD27+ peripheral memory B cells was reduced in HA patients with inhibitors compared to healthy controls [60]. Importantly, restoration of this population correlated with successful response to ITI [60], perhaps reflecting the re-establishment of "normal" memory B cells. Interestingly, non-class switched IgD+ B cells were prevalent among the remaining memory B cells in patients with inhibitors. Whether these cells are capable of responding to FVIII is unknown, but it is intriguing to speculate that the population of IgD+ memory cells in peripheral blood might represent a T-independent population of memory B cells similar to those found in MZ of spleen.

10. Lessons from Anti-CD20 Therapy

Many treatment centers have added Rituximab (anti-CD20) [61] to tolerogenic protocols used in acquired hemophilia in an effort to improve treatment efficacy. The anti-CD20 depletes both immature and recirculating mature B cells, most likely through a combination of antibody-dependent cellular cytotoxicity (ADCC), complement-mediated lysis, and/or by triggering apoptosis [62,63]. Rituximab has been reported to improve treatment for acquired hemophilia when used in conjunction with prednisone and/or cyclophosphamide [64], or when used as a second-line therapy to treat spontaneous relapse [65]. Rituximab has also been added to ITI therapy to treat congenital hemophilia patients with inhibitors. It has been suggested that rituximab treatment might establish tolerance faster than ITI alone [66]. Given the heterogeneity of hemophilia patient populations and the variations in treatment protocols, it is difficult to ascertain if treatment with rituximab is more effective than currently used ITI therapies. However, mechanistic insights into the memory cell response to FVIII can be gained from these studies.

Presumably, depletion of CD20+ cells results in the eventual exhaustion of the existing anti-FVIII plasma cell population in spleen and bone marrow and allows for repopulation of B cells tolerant to FVIII. Repopulation of the peripheral B cell population in patients with inhibitor antibody occurs

within six months to one year post rituximab treatment [66,67]. Experimental evidence strongly suggests this repopulation must occur concurrent with physiologically relevant levels of FVIII [68]. In patients whose inhibitor returned following rituximab treatment, the newly synthesized antibodies displayed changes in subclass distribution and epitope specificity compared to pre-rituximab antibodies, suggesting that they were derived from B cells that repopulated the periphery post-rituximab treatment, and not generated from an existing pool of long-lived plasma cells that escaped deletion [13,14]. Hence, lack of a long-term response to rituximab might represent a failure to re-establish peripheral tolerance to FVIII, rather than incomplete depletion of specific memory B cells. Of note, an early report regarding the efficacy of rituximab treatment in patients with inhibitors described its effects in a congenital hemophilia patient who had developed a concurrent anti-self FVIII autoantibody. Rituximab treatment resulted in a very rapid decline in autoantibody within two weeks, however, alloantibody production lingered for two months longer [69]. This result suggests that rituximab treatment may in fact be more effective for acquired hemophilia, and also lends credence to the idea that there are substantial differences in the development and maintenance of memory B cells in congenital hemophilia patients with inhibitors *vs.* acquired hemophilia patients.

It should be noted that depletion of B cells by anti-CD20 appears to be dependent on microenvironment, as splenic MZ B cells are more resistant to CD20 depletion [70]. Importantly this "sparing" of MZ B cells may be required to induce tolerance to FVIII following rituximab treatment [19]. Insight into this possibility may be gleaned from studies of lupus patients treated with rituximab. As is the case in HA patients with inhibitors, lupus patients also demonstrate varying lengths of disease regressions. An overabundance of post germinal center memory B cell expansion at the expense of MZ B cells was observed in patients with short disease remissions [71]. On the other hand, a predominance of "transitional" B cells (identified by expression of CD24 and CD28) during the reconstitution period was associated with patients who displayed prolonged disease remission. Other evidence indicates that selective repopulation of a regulatory IL-10 secreting population of memory B cells may be required to induce tolerance following rituximab therapy [72,73]. As comparative phenotyping of transitional, memory, and naïve B cell subsets has yet to be conducted in either HA inhibitor patients or acquired inhibitor patients, it remains to be determined which, if any, specific B cell subsets are required for successful induction of tolerance to FVIII.

11. Conclusions

More investigation is required regarding the role of memory B cells in the maintenance of anti-FVIII antibody responses. Substantial differences may exist between memory B cells that develop in congenital hemophilia patients with inhibitors and those in acquired hemophilia. Specifically, development of alloantibody to FVIII is T-dependent, and class-switching occurs in the germinal center. The size of the resulting memory B cell pool is unknown, but it is clear that successful immune tolerance requires the modification of these cells. In contrast, very little is known about memory B cells in acquired hemophiliacs, but it seems likely that these cells develop in the extrafollicular environment, and may rely less on T dependent response during their generation. They also appear to be more susceptible to depletion with rituximab. Future therapies for inhibitor antibodies should take

into account these possibilities, and further research may help to define specific survival signals that may be targeted to increase the likelihood of success in tolerogenic therapy.

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Author Contributions

A.M.A. researched and wrote sections specific for anti-CD20 treatment, memory B cells, and autoimmunity. C.K.H. researched and wrote sections specific for FVIII structure and function, ITI therapy, and development of cellular immune response. K.C.-S. supervised research, contributed to writing, and edited the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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