The mechanisms of immunopathology underlying B cell depletion therapy-mediated remission and relapse in patients with MuSK MG.

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ABSTRACT

In a series of studies, we applied reverse translational medicine, which affords understanding of immune pathogenesis via therapeutic intervention, to the MuSK subtype of myasthenia gravis (MG). Treatment with CD20-specific B cell depletion therapy (BCDT) demonstrated that MuSK MG patients respond remarkably well; the majority invariably reached remission accompanied by a remarkable drop in autoantibody levels. Circulating antibodies are primarily produced by bone marrow resident plasma cells, which do not express CD20. So, how does BCDT diminish MuSK autoantibodies and induce rapid remission? We developed a mechanistic model, which hypothesized that plasmablasts, which are short-lived antibody secreting B cell populations, produce MuSK-specific autoantibodies. Anti-CD20-mediated BCDT is expected to deplete CD20-expressing plasmablasts or CD20 expressing memory cells that supply the plasmablast population. To test this hypothesis, we performed a series of investigations, which were reported over the last seven years and are summarized in this review. First, we isolated plasmablasts from patients and generated human recombinant monoclonal autoantibodies (mAb) which bound MuSK and had pathogenic capacity, demonstrating that MuSK autoantibodies can be produced by this specific cell population. The characterization of the mAbs showed that MuSK autoantibodies can include unique properties including unusually high antigen binding affinity, and an elevated frequency of *N*-linked glycosylation in their binding domains. Further characterization suggested that MuSK autoantibody-producing cells may form in the early stages of B cell development due to defective tolerance mechanisms. Finally, we sought to determine how these pathogenic B cell clones behave over time. High throughput B cell receptor sequencing was applied to investigate longitudinally collected samples from patients treated with anti-CD20-mediated BCDT. MuSK-specific clonal variants were detected at multiple timepoints spanning more than five years and reemerged after BCDT-induced remission, predating disease relapse by several months. These collective investigations provide a more detailed mechanistic understanding of MuSK MG, the key features of which include the production of autoantibodies by circulating plasmablasts that can be diminished by CD20-specific BCDT, but a subset of which persist which then seed a reemergence of pathogenic clones prior to manifestation of clinical relapse.

Key Words: Myasthenia gravis, muscle-specific tyrosine kinase (MuSK), B cells, autoantibodies, B cell depletion therapy, rituximab, tolerance, reverse translational medicine, remission, relapse, longitudinal specimen collection, immunomechanisms, mechanistic model

Introduction

Autoimmune myasthenia gravis is an archetypal autoantibody-mediated disease (1, 2). The autoantibodies target molecules at the neuromuscular junction (NMJ), which leads to increased fatigability and muscle weakness in patients (1, 2). Disease subtypes can be defined by autoantibody specificity. The most frequently observed MG subtype is characterized by autoantibodies against the nicotinic acetylcholine receptor (AChR), comprising approximately 85% of patients (1). The remaining patients can harbor autoantibodies targeting muscle-specific kinase (MuSK) (3) or lipoprotein receptor-related protein 4 (LRP4) (4, 5), while a small fraction do not have detectable circulating autoantibodies to known targets. Accordingly, this group is collectively categorized as seronegative. The pathogenic capacity of autoantibodies targeting AChR and MuSK have been clearly demonstrated with both *in vitro* (6-11) and *in vivo* approaches (12).

The immunopathology of the subtypes can differ substantially, which is well highlighted by the AChR and MuSK subtypes. The immunopathology of AChR MG is mediated by IgG1 and IgG3 subclass autoantibodies, which effect disruption of AChR signaling through complement activation and subsequent tissue damage, initiating receptor internalization, and interfering with ACh binding. Conversely, MuSK MG is largely governed by IgG4 autoantibodies. These autoantibodies are ineffective in activating complement and mediate pathology by physically blocking NMJ protein-protein interactions. Specifically, MuSK Abs inhibit the interaction between MuSK and LRP4, which is essential for MuSK phosphorylation and subsequent effective AChR clustering and signaling (13). Moreover, the pathogenic capacity of MuSK autoantibodies is partly dependent upon fragment antigen-binding (Fab)-arm exchange, which generates functionally monovalent IgG4 antibodies (14).

While much of the underlying immunopathology of MuSK MG is understood, further details are needed. Over the last decade, we established a potential mechanism describing how pathogenic autoantibodies develop in MuSK MG through applying reverse translational medicine. That is, by using knowledge observed in clinical studies in combination with basic immunological research (15, 16). Spe-

cifically, we leveraged the positive effect of anti-CD20-mediated B cell depletion therapy (BCDT) in treating MuSK MG patients, to build a model in which CD20-expressing plasmablasts are the key disease-relevant cells that produce MuSK autoantibodies (17). We pursued testing of this model and further investigated the immunopathology of relapse that can occur following anti-CD20-mediated BCDT-induced remission in MuSK MG patients (17). This mini-review will focus on different aspects of the immunopathology of MuSK MG and will provide insights into the immunopathology of relapse after CD20-mediated BCDT.

What we learned from anti-CD20-mediated B cell depletion in MuSK MG – the basis of our mechanistic model.

B cells express different surface markers at different stages of B cell development and these markers can be used to identify and target specific B cell subsets (18). The cluster of differentiation molecule 20 (CD20) is not expressed on B cells at early stages of development or when they have differentiated to plasma cells (18). Targeting CD20 with the monoclonal antibody, rituximab (RTX), was first successfully used for the treatment of B cell malignancies (19-21). Rituximab was then shown to be effective in autoimmune diseases including antibody-mediated chronic inflammatory demyelinating polyneuropathy (CIDP), pemphigus vulgaris, multiple sclerosis, rheumatoid arthritis (22-25), and MuSK MG, first in 2008 by the research group of Isabel Illa (26), then shortly afterward in a number of corroborative studies (17, 27, 28), including several by our group at Yale (29, 30).

The B cell subsets that secrete autoantibodies (31) are short-lived plasmablasts and plasma cells. Some plasmablasts may express low levels of CD20, while plasma cells do not express CD20 (18, 32, 33). The response to RTX observed in MuSK MG patients often includes a rapid and near-complete reduction of autoantibody titer and subsequent disease remission. The Illa group elegantly demonstrated that, in contrast to the MuSK autoantibody titer, both total circulating IgG and tetanus vaccine specific IgG titers did not significantly diminish after BCDT (17). A sensible hypothesis explaining these findings is that the observed effect was based on the depletion of MuSK autoantibody-expressing, CD20-positive, short-lived plasmablasts and/or CD20-positive memory B cells that supply this plasmablast population (16). To test this mechanistic hypothesis, we isolated plasmablasts from MuSK MG patients with the intent of determining whether they produced MuSK specific autoantibodies (34). We took considerable care in the flow cytometry-based isolation, as these cells are challenging to identify because they are rare within the circulation and share surface markers with other B cell subsets. The additional step of examining the isolated cells via morphology was performed, as plasmablasts are distinctly bigger than naive or memory B cells due to an enlarged cytoplasm. These isolated plasmablasts were cultured in a manner that allowed for antibody secretion into culture media, which was then tested for binding specificity towards MuSK using a live cell-based assay (34). We found that the secreted antibodies bound to MuSK demonstrating that plasmablasts are a source of autoantibodies in MuSK MG (34).

To perform a more rigorous experimental demonstration, we next produced recombinant human MuSK monoclonal autoantibodies (mAbs) from these plasmablasts (33-35). We also included experienced (memory) B cells in our cell isolation approach; the result of which was that most of our MuSK mAbs originated from plasmablasts, while the rest were derived from memory B cells (33-35). Recombinant production of human mAbs allowed for an unlimited source of human autoantibodies for study, given that those secreted in the culture media by stimulated B cells are limited in quantity. Additionally, experiments could be performed with individual autoantibody clones rather than a heterogeneous mixture found in the bulk cell culture media or serum. In addition to validating binding properties, we leveraged these mAbs to further investigate the development of pathogenic B cells in MuSK MG and the pathogenic effect of autoantibodies at the NMJ.

Development of autoantibodies in MuSK MG

Human serum contains a multitude of distinct antibodies with different variable regions, which is vital for the broad reactivity to a vast array of potential pathogens (36). Although broad reactivity is important for protection against foreign antigens, self-reactivity is a possible by-product of the process that generates a diverse B cell and serum antibody repertoire. This is because random combinations of antibody variable region genes are assembled to produce a repertoire with many different antigen specificities during B cell development. However, that initially generated repertoire can include reactivity to self (37). Both central and peripheral tolerance checkpoints prevent these self-reactive B cells from further development (38, 39). The fidelity of these checkpoints is compromised in several autoimmune disorders. The result of which is increased frequencies of self-reactive B cells within the naïve B cell repertoire (40). We found that the central and peripheral tolerance checkpoints are defective in MuSK MG (41). Therefore, it is reasonable to propose that the development and origin of MuSK autoantibodies is partly due to unsuccessful counter-selection of self-reactive B cells due to tolerance defects.

The MuSK mAbs that we (33-35) and others (42) generated contain multiple mutations in the sequences of their variable region, which is the characteristic hallmark of the affinity maturation process. The reversion of these sequences to their corresponding germline-encoded form, which would be found in the naïve B cell precursors, is a common approach that is used to investigate the development or origin of autoantibodies (43, 44). Given that some small

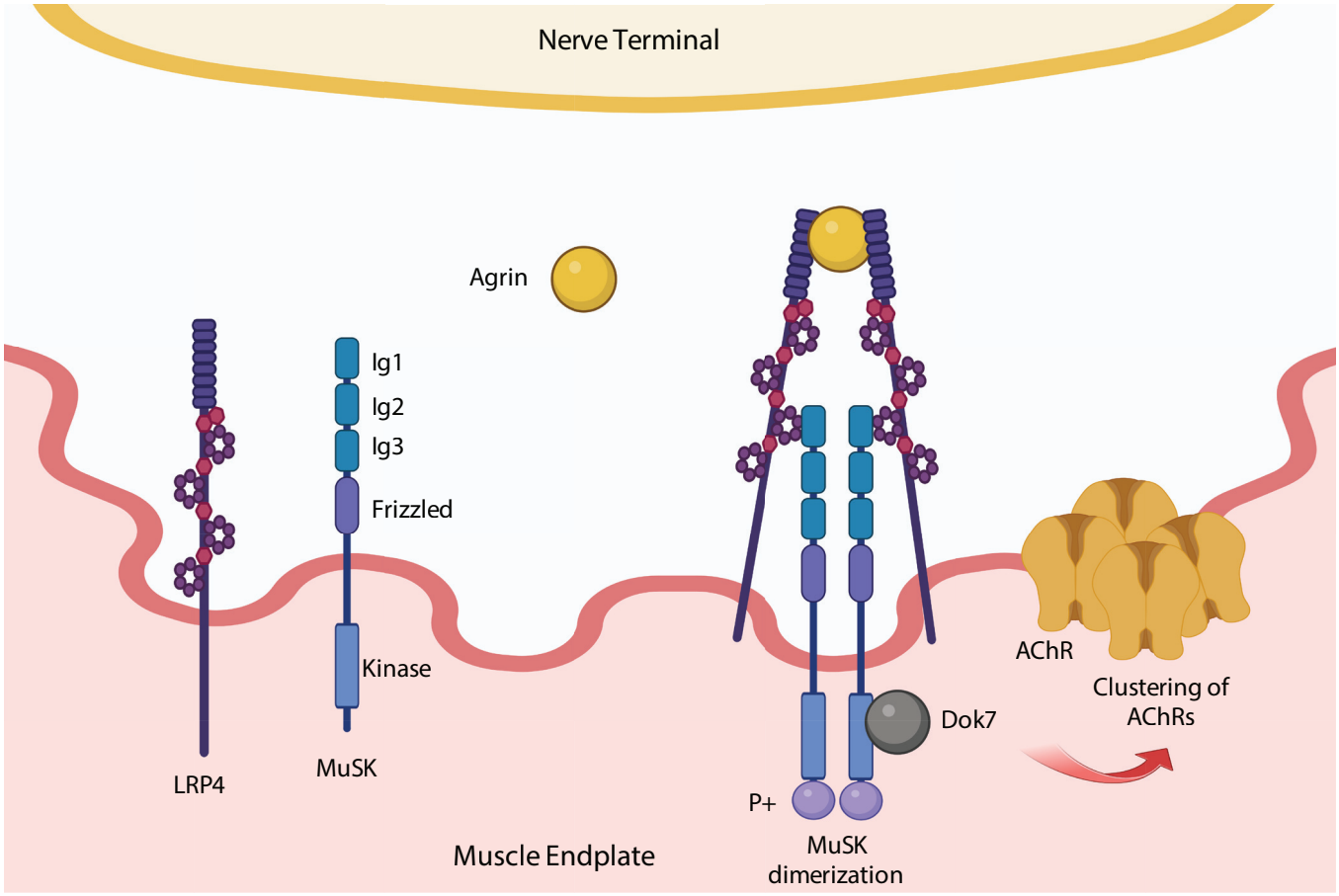

Figure 1. Schematic of the interaction of MuSK, LRP4, agrin and Dok7 at the neuromuscular junction.
The MuSK/LRP4 pathway is involved in the clustering of AChRs at the neuromuscular junction. MuSK has three immunoglobin-like domains 1-3 (Ig1-3) and a cysteine-rich domain (frizzled domain) on the ectodomain and an intracellular tyrosine kinase domain (65, 66). LRP4 is the (membrane-bound) ligand of MuSK and binds to the Ig-like domain 1 (54). The interaction of MuSK and LRP4 is enhanced when agrin binds to LRP4 which changes its conformation (54). Downstream of kinase-7 (Dok7) is an intracellular activator and substrate of MuSK, which binds to the kinase domain (65). Dok7 facilitates the autophosphorylation of MuSK (65, 67). The activation of the MuSK/LRP4 pathway results in the dimerization and autophosphorylation of MuSK, which is important for the activation of downstream pathways that lead to the clustering of AChRs at the NMJ. LRP4 = Low Density Lipoprotein Receptor-Related Protein 4; MuSK = muscle-specific tyrosine kinase; AChR = nicotinic acetylcholine receptor. P+ = phosphorylation. This figure was created with Biorender.com.

sequence areas of the antibody variable region (namely parts of the complementary determining region 3 (CDR3)) are not encoded by gene segments, the best approximation of the naïve, unmutated sequence is commonly called the unmutated common ancestor (UCA). Testing the binding properties of UCAs to the antigen recognized by the mature form can lead to at least two potential outcomes. The first is that UCA antibodies recognize the antigen, suggesting that the parental naïve B cell bound the antigen and that the same self-antigen is driving the affinity maturation process. The second outcome is that UCA antibodies do not recognize the antigen, suggesting that the mature B cell may gain antigen specificity during the affinity maturation process. UCAs in several autoimmune diseases have been investigated; there is no clear conclusion whether autoantigens predominantly drive the development of autoantibodies or whether antigen reactivity develops during affinity maturation. UCA autoantibodies in neuromyelitis optica spectrum disorder (NMOSD), pemphigus vulgaris (PV) and systemic lupus erythematosus (SLE) do not recognize the associated self-antigen (43, 45-47), whereas UCAs in rheumatoid arthritis and other mAbs in PV can exhibit specific reactivity to the disease-associated self-antigen (48, 49). We found that UCAs of MuSK mAbs recognize MuSK (33, 44) and that these UCAs have strikingly high affinities (nanomolar) for MuSK (44). Thus, we speculate that MuSK might be both the initiating and affinity maturation-driving self-antigen of MuSK specific B cells, and that they escaped elimination as a consequence of defective tolerance mechanisms.

Pathogenic and functional properties of MuSK autoantibodies

Understanding the role of MuSK is an essential prerequisite for investigating how pathogenic MuSK autoantibodies interfere with neuromuscular signaling at the NMJ **(Figure 1)**. MuSK is associated with the development and preservation of the NMJ (50-53) and it forms a functional unit with low density lipoprotein receptor-related protein

4 (LRP4) (54). The activation of the MuSK/LRP4 pathway results in the dimerization and autophosphorylation of MuSK, which is important for the activation of downstream pathways that lead to the clustering of AChRs at the NMJ **(Figure 1)** (51). Most serum-derived MuSK autoantibodies recognize the Ig-like domain 1 of MuSK, which interacts directly with LRP4 **(Figure 1)** (54, 55). It has been demonstrated, with both *in vitro* and *in vivo* approaches, that MuSK autoantibodies prevent the interaction of MuSK and LRP4, which leads to diminished clustering of AChRs and subsequent impaired neuromuscular signaling (14, 33, 42, 56-58). Some of the MuSK mAbs that we generated (33-35), specifically recognized the Ig-like domain 1 (33) while several others recognized the Ig-like domain 2 (35). Irrespective of their domain specificity, these mAbs reduced AChR clustering when tested with an *in vitro* approach (33, 35).

IgG4 subclass antibodies have a unique property in that they can exchange half-molecules with other IgG4 subclass antibodies during a process termed Fab-arm exchange (FAE), which produces bispecific IgG4 that bind to their target antigen in a monovalent manner (59, 60). MuSK MG autoantibodies are mainly of the IgG4 subclass (61-63) and functional monovalency potentiates their pathogenic effect at the NMJ (14, 42, 44, 64). In work we performed collaboratively with Angela Vincent and Michelangelo Cao (35), we found that recombinant divalent MuSK mAbs phosphorylate MuSK and reduce AChR clusters in comparison to non-disease relevant, control antibodies. In contrast, monovalent variants of these same antibodies are much more pathogenically potent because they robustly diminish AChR clustering (44). Given these observations, we proposed that divalent antibodies can crosslink and activate MuSK **(Figure 1)**. Monovalent antibodies, in contrast, block the interaction of MuSK with LRP4 without any artificial crosslinking of MuSK. Thus, our work, along with key findings from the Leiden University group led by Maartje Huijbers and Jan Verschuuren (14, 42), demonstrate that monovalency - generated by IgG4 FAE - is important for the pathogenic effect of MuSK autoantibodies at the NMJ.

In addition to valency, we found that affinity is important for the pathogenic capacity of MuSK autoantibodies (44). We found that only monovalent Fabs of mature, mutated autoantibodies prevented agrin-induced clustering of AChRs, while UCA Fabs did not show any pathogenic capacity despite having high affinities (nanomolar range) for MuSK (44). Thus, we hypothesized that binding kinetics (association and dissociation) may play a key role in the different pathogenic capacities. To investigate this further, we turned to affinity measurements. Our autoantibodies recognize MuSK over a wide range of concentrations when using live cell-based assays (CBAs) (35, 44). However, the static nature of these assays does not provide any information on the kinetics of antibody association and dissociation. Consequently, CBAs are not ideal for properly measuring affinity. Accordingly, we used bio-layer interferometry and monovalent Fabs to measure the affinity of our antibodies to MuSK, rather than divalent mAbs, which would have provided avidity values. We found that mature MuSK autoantibodies had exceptionally high affinities (sub-nanomolar) and that the high Ka was driven by fast association and slow dissociation whereas their UCA counterparts associated slower and dissociated faster (44). Thus, high affinity, characterized by rapid association and delayed dissociation, together with monovalency appear to be key properties for the pathogenic development of MuSK mAbs and are necessary for potent monovalent pathogenic capacity at the NMJ (44).

Unique features of the circulating B cell repertoire in MuSK MG

We next turned our attention to studying the B cells in MuSK patients. We started by examining the BCR repertoire using adaptive immune receptor repertoire (AIRR) sequencing. Although conspicuous changes in the overall repertoire of MuSK MG patients relative to healthy controls were not observed, we observed some unique abnormalities (68). These changes in the B cell repertoire in MuSK MG are subtle but seem to be specific as the repertoire of AChR MG showed different abnormalities (68). The B cell repertoire of MuSK MG shows differences in preferential usage of variable region gene segments and indicates impaired mechanisms of central tolerance during B cell development (68). The most conspicuous observation provided by the BCR repertoire analysis concerned the frequency of *N*-linked glycosylation site motifs (N-X-S/T, X cannot be proline) in the antibody variable region (IgG-$V^{N\text{-}Glyc}$). The frequency of IgG-$V^{N\text{-}Glyc}$ is elevated in AChR and MuSK MG in comparison to healthy individuals (42, 69). These glycosylation sites were either acquired through affinity maturation or present due to a preferential usage of the select gene segments containing glycosylation sites in their germline configuration (69). Several of our MuSK mAbs included IgG-$V^{N\text{-}Glyc}$ motifs affording us the opportunity to test whether they were involved in binding, given their conspicuous occupation of the variable region. The removal of these glycosylation sites, however, did not alter the binding capacities of these mAbs (42, 69). Thus, the functional purpose of *N*-linked glycosylation sites in the variable region of autoantibodies in MG is currently not understood but might be connected to altered B cell activation (70).

Immunomechanisms underlying relapse after anti-CD20-mediated B cell depletion

While most MuSK patients reach clinical remission following anti-CD20-mediated B cell depletion, patients can experience relapse years later (17, 71). Therefore, we wanted to study the immunomechanisms underlying

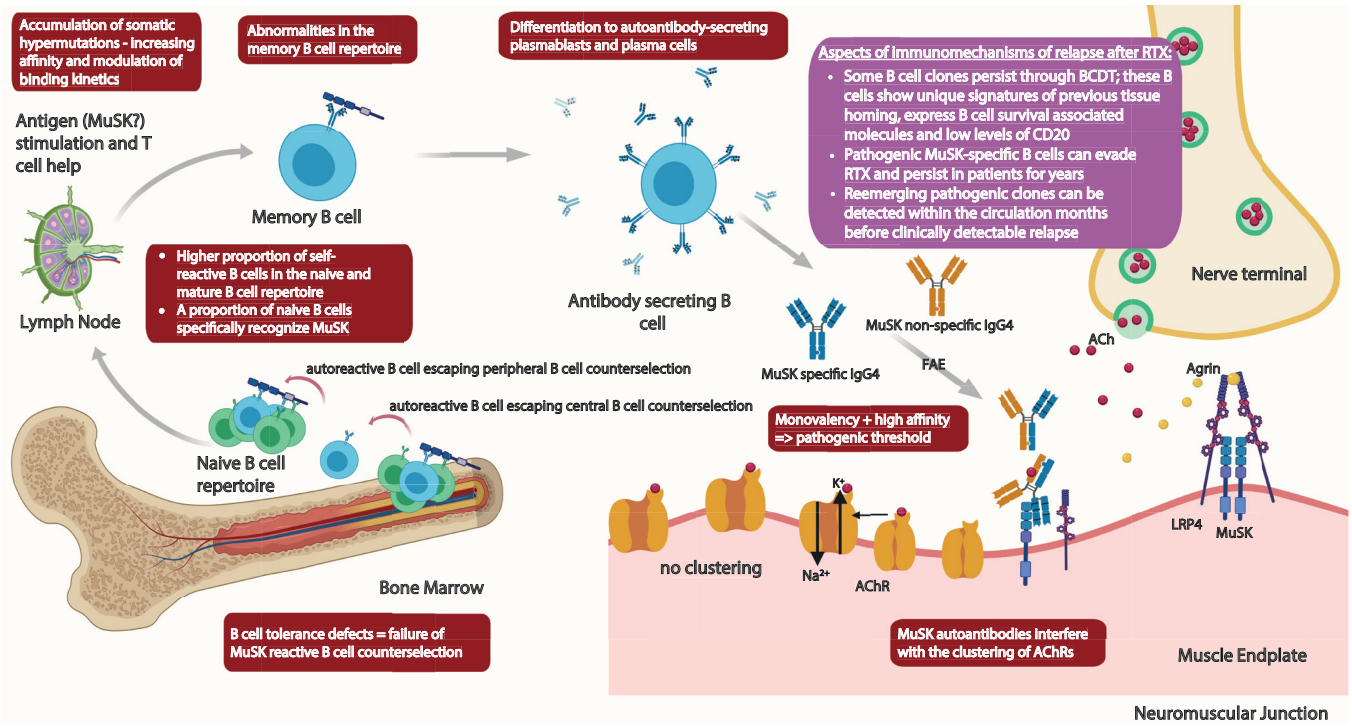

Figure 2. Schematic diagram showing the development of the pathogenic B cell repertoire and features of autoantibodies that mediate disease in MuSK MG. This figure was created with Biorender.com.

these relapses. We specifically focused on whether relapse is the consequence of reemerging historic clones or the development of newly generated pathogenic clones. To that end, we leveraged our MuSK mAbs, longitudinally collected samples, and AIRR sequencing. Specifically, with the BCR sequence of validated MuSK mAbs in-hand, we used AIRR sequencing to search for related clones present in longitudinal samples collected over several years prior to the mAb isolation. These longitudinal samples were collected during periods of both BCDT-induced remission and relapse. We found one pathogenic mAb and its corresponding clonal variants in a patient who had received several cycles of anti-CD20-mediated BCDT over almost 79 months (33). These clonal variants acquired changes in the antibody variable region sequence indicative of continuous affinity maturation in germinal centers; these changes did not alter the binding and pathogenic properties of the identified MuSK clone (33). The clonal variants reemerged before clinically-detectable relapse, concurrent with increasing MuSK autoantibody titer (33).

These persistent B cells express low levels of CD20 and show expression signatures associated with previous tissue homing and B cell survival (32). Likewise, plasmablast populations examined at the time of relapse expressed molecular signatures associated with B cell survival, B cell proliferation, and tissue homing (32, 33). Anti-CD20-mediated BCDT, however, is effective in eliminating antigen specific B cells in the lymph nodes in NMOSD (72), and decreases the levels of B cells in both the circulation and bone marrow in RA (73). Thus, it is not clear whether tissue homing is protective or indicative of recent repopulation and proliferation in germinal centers.

Summary

Over the last decade, we developed a model to describe the development of pathogenic B cells in MuSK MG **(Figure 2)**: The proportion of self-reactive B cells is elevated in the naïve B cell repertoire due to defects in the central and peripheral tolerance checkpoints (41). Among these self-reactive naïve B cells are clones that show strong and specific binding to MuSK indicating that the MuSK antigen might be initiating B cell activation and may also drive affinity maturation of these B cells in germinal centers (44), followed by differentiation into antibody-secreting plasmablasts (34). The secreted antibodies are mostly of the IgG4 subclass (61-63) and become functionally monovalent through the process of Fab-arm exchange (64). Binding of the monovalent pathogenic mAbs to MuSK impedes the clustering of AChRs which impairs the signaling from the nerves to the muscles (14, 33, 42, 44). Thus, affinity maturation and monovalency are necessary for the pathogenic development of MuSK autoantibodies and their pathogenic capacity at the NMJ (14, 33, 42, 44, 64). Characteristic abnormalities in the B cell repertoire of MuSK MG patients include the elevated frequency of *N*-linked glycosylation motifs within the variable region (68, 69); the functional relevance of these observations is the object of future investigations. Lower expression of CD20 on persistent B cells, together with molecular signatures associated with B cell survival and tissue homing

(32), may contribute to survival of persistent clones during BCDT as well as continuous antigenic stimulation. Among these persistent clones are pathogenic B cell clones that can be traced longitudinally over several years and through continuous BCDT treatments (33). These pathogenic clones can reemerge months before noticeable clinical relapse together with increasing autoantibody levels (33). Overall, this body of research provides both a mechanistic understanding of MuSK MG immunopathology and how disease relapse develops during a commonly used treatment strategy.

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Competing interests
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