Journal of Neuroimmunology 337 (2019) 577080

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Review Article

Myasthenia gravis: State of the art and new therapeutic strategies

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ARTICLE INFO

Keywords:
Myasthenia gravis
Acetylcholine receptors
Neuromuscular junction
Steroids
Immunosuppressant drugs
Rituximab
Eculizumab

ABSTRACT

Myasthenia Gravis (MG) – an autoimmune neuromuscular disease – is known by the production of autoantibodies against components of the neuromuscular junction mainly to the acetylcholine receptor, which cause the destruction and compromises the synaptic transmission. This disease is characterized by fluctuating and fatigable muscle weakness, becoming more intensive with activity, but with an improvement under resting. There are many therapeutic strategies used to alleviate MG symptoms, either by improving the transmission of the nerve impulse or by ameliorating autoimmune reactions with e.g. steroids, immunosuppressant drugs, or monoclonal antibodies (rituximab and eculizumab). Many breakthroughs in the discovery of new therapeutic targets have been reported, but MG remains to be a chronic disease where the symptoms are kept in the majority of patients. In this review, we discuss the different therapeutic strategies that have been used over the years to alleviate MG symptoms, as well as innovative therapeutic approaches currently under study.

1. Introduction

Myasthenia Gravis (MG), a rare autoimmune disease, is characterized by fluctuating muscle weakness and fatigue attributed to the impaired synaptic transmission at the neuromuscular junction (NMJ) (Berrih-Aknin et al., 2014; Gilhus et al., 2016). Dysfunction at NMJ is caused by autoantibodies that target specific antigens of the postsynaptic muscle end-plate components. The autoimmune response, in most patients, is mediated by antibodies against the acetylcholine receptor (AChR). In about 5% of the patients, the autoreactive antibodies are directed against a protein known as muscle specific tyrosine kinase (MuSK) that has a crucial role in the clustering of AChRs. In a minor proportion of MG patients, the agrin receptor (low-density lipoprotein receptor-related protein 4 - LRP4) has been recently identified as a new autoantigenic target (Burden et al., 2013; Evoli et al., 2018; Verschuuren et al., 2013). Based on the differences in clinical setting, age at onset and autoantibody profile, and by the presence or absence of thymic pathology, several MG clinical subtypes have been identified (Koneczny and Herbst, 2019). The autoantibodies, i.e. the IgG subclasses, are instrumental for subgrouping MG patients, as they are sensitive and specific diagnostic biomarkers and immunopathogenic factors, allowing an ideal diagnosis and treatment (Koneczny and Herbst, 2019; Zisimopoulou et al., 2013).

The knowledge of these different subtypes offers the clinicians the possibility to adapt the patients' treatment to their disease subgroup by the use of specific drugs to treat symptoms. But, patients that are not satisfied with the symptomatic treatment alone, should start the immunosuppressive treatment, knowing that the dose and drug

Abbreviations: ACh, Acetylcholine; AChR, Acetylcholine Receptor; aHUS, Atypical Hemolytic Uremic Syndrome; APC, Antigen Presenting Cell; CT, Computerized Tomography; DAF, Decay-Accelerating Factor; EPP, Endplate Potential; gMG, generalized Myasthenia Gravis; HLA, Human Leukocyte Antigen; IVIg, Intravenous Immunoglobulin; LRP4, low-density lipoprotein receptor-related protein 4; MAC, Membrane Attack Complexes; MG, Myasthenia Gravis; MuSK, Muscle Specific Tyrosine Kinase Receptor; NMJ, Neuromuscular Junction; PNH, Paroxysmal Nocturnal Haemoglobinuria; RA, Rheumatoid Arthritis; RyR, Ryanodine Receptor; TFH, T Follicular Helper cells; Tregs, Regulatory T cells; VGCCs, Voltage-Gated Calcium ion Channels; VGSCs, Voltage-Gated Sodium ion Channels

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https://doi.org/10.1016/j.jneuroim.2019.577080
Received 3 September 2019; Accepted 4 October 2019

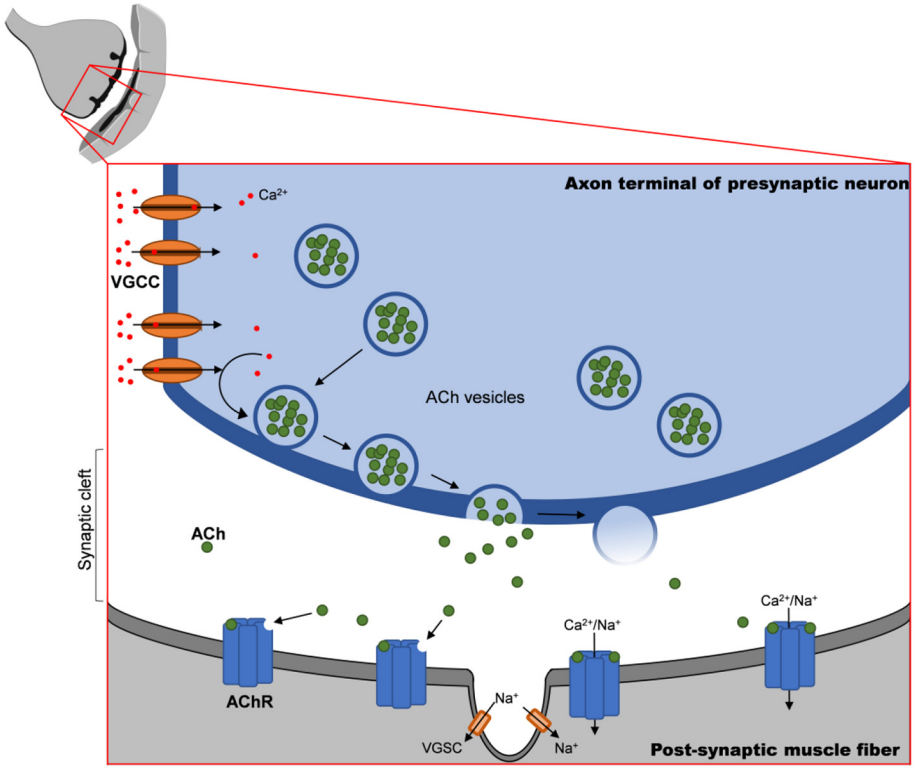

Fig. 1. Schematic representation of communication from neuron to muscle fibre in NMJ. As the action potential reaches the axon terminal of the presynaptic neuron, voltage-gated calcium channels (VGCC) are activated, allowing an influx of Ca^{2+} that triggers the translocation of acetylcholine (ACh) vesicles to the membrane, its fusion with subsequent release of ACh into the synaptic cleft. This neurotransmitter, once released diffuses and binds to the specific receptors in the postsynaptic muscle fibre. These are mainly nicotinic ACh receptors, (nAChRs), which are non-selective cation channels enabling the influx of cations, mainly Ca^{2+} and Na^{2+}, initiating a cascade of events in the muscle cell (Chandhok and Soh, 2016; Gundisch and Eibl, 2006; Ono, 2008; Campanari et al., 2016).

combination is not equal to all patients (Gilhus and Verschuuren, 2015). The NMJ is known to be the site of synaptic transmission of motor neurons and muscle fibres. Motor neurons, linked to the muscle tissue, are divided into numerous branches to innervate individual muscle fibres, allowing the conduction of electrical signals (action potentials) to the skeletal muscles. The nerve terminal contains vesicles that are responsible for the storage of the ACh neurotransmitter being particularly concentrated next to the presynaptic membrane, which is controlled by a transmembrane voltage-gated calcium ion (Ca^{2+}) channels (VGCCs). The motor endplate is the cell membrane adjacent to the presynaptic terminal above the synaptic cleft, and has deep junctional folds equipped with transmembrane voltage-gated sodium ion (Na^{+}) channels, as well as many other proteins and proteoglycans (agrin, rapsyn, LRP4, muscle specific tyrosine Kinase - MuSK and also acetylcholine receptors - AChRs). ACh diffuses through the synaptic cleft to the motor endplate, binding to AChRs, which are mainly nicotinic AChRs, and thus creating an initial influx of Na^{+} into the muscle fibre - called endplate potential (EPP) - which causes muscle fibre depolarization, activation of the voltage-gated sodium ion (Na^{+}) channels (VGSCs) in the junctional folds thereby creating a further influx of Na^{+} (Fig. 1). In healthy NMJ, the amplitude of EPPs generated by synaptic transmission overcomes the threshold needed to generate an action potential in the muscle. If the EPP falls below the threshold level, there is no action potential. The reduction in this neuromuscular transmission is therefore the electrophysiological defect responsible for the MG symptoms, as well as the electrophysiological findings that are identified in clinical diagnosis (Querol and Illa, 2013; Conti-Fine et al., 2006).

2. Epidemiology, signs and symptoms and pathophysiology of myasthenia gravis

2.1. Epidemiology

Autoimmune MG has an annual incidence of 4–12 per million people, with a low prevalence of 40–180 per million people attributed to the improvements in diagnostic tools, efficiency of treatment, as well as to the increased longevity of the population in general (Koneczny and Herbst, 2019; Phillips, 2004; Carr et al., 2010). AChR-associated MG has a bimodal age pattern of incidence, i.e. it has a peak in young adults (aged about 30 years old) followed by a constant increase in incidence with advancing age (older than 50 years old) (Heldal et al., 2009). Based on sex, the incidence peak in young adults is more frequent in women, while the late-onset MG is slightly more frequent in men (Carr et al., 2010). Epidemiological data indicate that LRP4-associated MG is half in the same proportion of the MuSK form of the disease. The MuSK-associated MG incidence is estimated to be about 0–3 patients per million per year with a prevalence of 2–9 per million people, being more frequently found in Northern Europe rather than in the South (Guptill et al., 2011). Some geographical variation of this disease is also seen, as well as in its subtypes, which can be explained by the genetic predisposition and also by external factors linked to infections or diet.

2.2. Signs and symptoms

The major symptom of MG is known to be the muscle weakness involving susceptible muscle groups. Some characteristics, such as the combination of weakness localization, variation in weakness over time and exercise-induced weakness, allow the diagnosis of the disease for all subgroups (Querol and Illa, 2013; Meriggioli and Sanders, 2009; Skeie et al., 2010). Weakness fluctuates from day to day or hour to hour, getting worse with activity but improving upon resting. Patients present several degrees of ptosis, diplopia, dysarthria, dysphagia and dyspnea, as well as facial weakness or fatigable limbo or axial weakness. Usually, MG is also characterized by ocular weakness, which is expressed as fluctuating ptosis and/or diplopia, being diagnosed in about 85% of patients. MG progression to generalized weakness usually occurs in a period of 2 years from the onset of the disease. Weakness of facial muscles is fairly frequent and when the patients are examined carefully, most of them exhibit detectable weakness of eyelid closure, with/without lower facial weakness. The course of MG varies, in many patients exhibiting intermittent worsening of symptoms triggered by

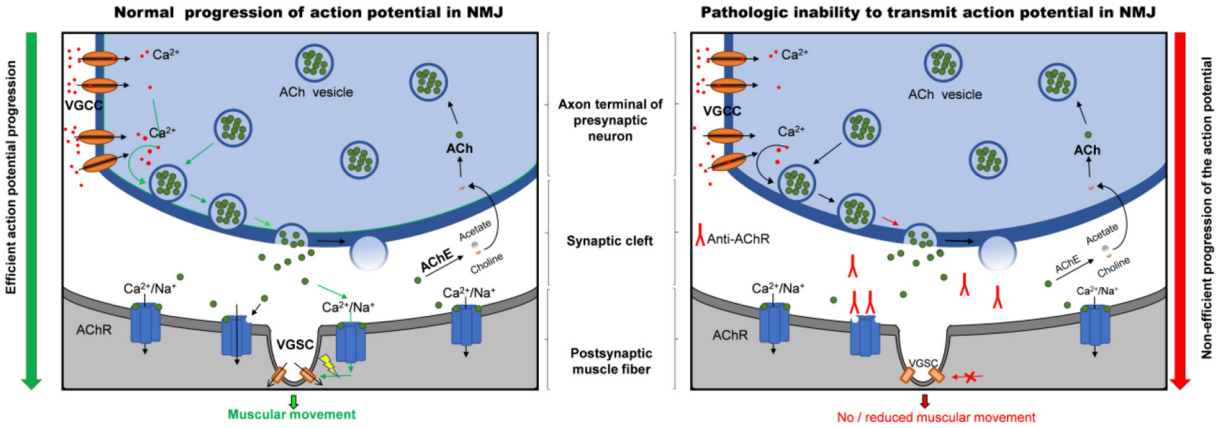

Fig. 2. NMJ in physiological state (left) and in MG pathophysiology (right). In MG pathology, the presence of anti-AChR antibodies leads to the destruction and reduction of AChR number in post-synaptic muscle membrane hindering (or even preventing) the propagation of the action potential reducing (or preventing) muscle contraction.

infections, emotional stress, surgeries and medication. This is shown especially during the first year upon the onset of the disease and the progression to maximum severity is observed over the first 2 years of onset. Spontaneous long-lasting remissions are uncommon (in 10–20% of patients) (Grob et al., 2008). The loss of functional AChRs, in MG, causes a failure in neuromuscular transmission, the basis of most symptoms.

2.3. Pathophysiology

The pathogenic role of anti-AChR antibodies in MG has been clearly described, having three main mechanisms underlying the loss of functional AChRs (Drachman et al., 1980), being the lysis of the muscle endplate, mediated by complement proteins, the most common, causing a morphological damage in the postsynaptic muscle membrane. This results in a simplification and also in a distortion of the normal folded pattern of the postsynaptic membrane, which has consequences on AChRs signalling, causing a decrease in the number of voltage-gated Na^+ channels and creating an increased threshold needed for the generation of the muscle fibre action potential (Ruff and Lennon, 2008). Considering that, in a healthy NMJ (Fig. 2), the EPPs amplitude generated by synaptic transmission exceeds the required threshold to generate an action potential in the muscle, to assure that an action potential is always generated, and muscle contraction occurs, there is an excess of potential generated. The safety factor is thus described as the ratio between the actual EPP and the threshold potential required to generate an action potential in the muscle.

The complement cascade system has a crucial role in innate and also antibody-mediated immunity, since it operates towards the protection of the host against infection through the recognition and elimination of microorganisms. In addition, it can also remove the immune complexes and modified/dead self-cells, where the triggering is sequential and extremely regulated by surface membrane regulators (CD55 - decay-accelerating factor (DAF) and also CD59 - protectin) (Drachman, 1978; Zipfel and Skerka, 2009). The classical pathway is triggered in anti-$AChR^+$ MG, starting with complement protein C1q binding to the antigen/antibody complexes (immunoglobulin (Ig)G1 or IgG3 autoantibodies bound to AChRs), which activates a cascade of events converging with the cleavage of complement protein C5 (which leads to the formation of pro-inflammatory and cell-lytic signal), with subsequent recruitment of C6, C7, C8, and multiple C9 units to form the membrane attack complexes (MACs) at NMJ which destroy their components and impair the neuron transmission (Howard Jr., 2018). Secondly, the accelerated internalization and the degradation of AChRs generated through the cross-linkage of AChRs by divalent antibodies have consequences in the loss of AChRs (Heinemann et al., 1977). And finally, direct blockade of AChRs by antibodies linked to acetylcholine-binding sites has shown to be significant in some patients (Burges et al., 1990). Pathogenic anti-AChR antibodies, from subclasses of IgG1 and IgG3, are known to have high affinity to the extracellular region of AChR, which synthesis requires the interaction of activated $CD4^+$ T cells with B cells. This interaction can prove the task of thymus in the formation of anti-AChR antibodies as it exhibits AChRs in its muscle cells. Fig. 3 shows the postsynaptic apparatus involved in the AChR clustering and target proteins in MG.

Muscle-specific tyrosine kinase (MuSK) - transmembrane endplate polypeptide - is implicated in a signalling pathway that controls the AChR clustering and the preservation of the normal functional integrity of the NMJ. MuSK antibodies are overall IgG4, which cannot activate the complement and therefore can also not induce antigen internalization, but can generate MG by direct inhibition of protein function (Koneczny et al., 2017). It is believed that the main effector mechanism of the antibody is the interference with MuSK-LRP4 binding and consequent dispersal of AChR clusters (Huijbers et al., 2013). Literature reports few studies that have analyzed lymphocyte responses in $MuSK^+$ MG. In the reported cases, the patients exhibited an enhanced frequency of $CD4^+$ T cells with inflammatory phenotype and a reduced rate of IL-10-producing B regulatory (B10) cells, in comparison to the controls (Guptill et al., 2015). Most of the patients with MuSK antibodies share the human leukocyte antigen (HLA) *DQ5* allele (Marino et al., 2014). In another study, MuSK IgG4 was reported to interfere with the binding between collagen-Q and MuSK (Kawakami et al., 2011). LRP4 was identified as the postsynaptic receptor for nerve derived agrin (which is a heparan sulphate proteoglycan released from the motor nerve terminal) and thereby a trigger of MuSK (Kim et al., 2008). LRP4 antibodies (especially IgG1) inhibit the agrin-LRP4 interaction, as well as the AChR clustering induced by agrin (Higuchi et al., 2011; Zhang et al., 2012). This pathological mechanism, i.e. blocking of agrin signalling, is certainly one of the most credible approaches for LRP4 antibodies (Kummer et al., 2006), as impaired agrin signalling leads to less stable NMJs, with more dispersed and un-clustered AChRs. Other pathophysiological mechanisms of LRP4 antibodies cannot be excluded, mainly the blocking of the LRP4-MuSK interaction or a role for complement which is implied by the involvement of the IgG1 autoantibodies. Agrin antibodies can be detected even in a few patients with MG with/without antibodies against AChR, MuSK or LRP4, but these antibodies have been detected only in patients with MG which led to the conclusion that they are specific to the disease (Gasperi et al., 2014; Zhang et al., 2014; Witzemann et al., 2013). It is known that agrin is responsible for the regulation of the formation, maintenance and regeneration of the NMJ. Interference with agrin function leads to poor neuromuscular transmission. No direct pathogenic effect of agrin

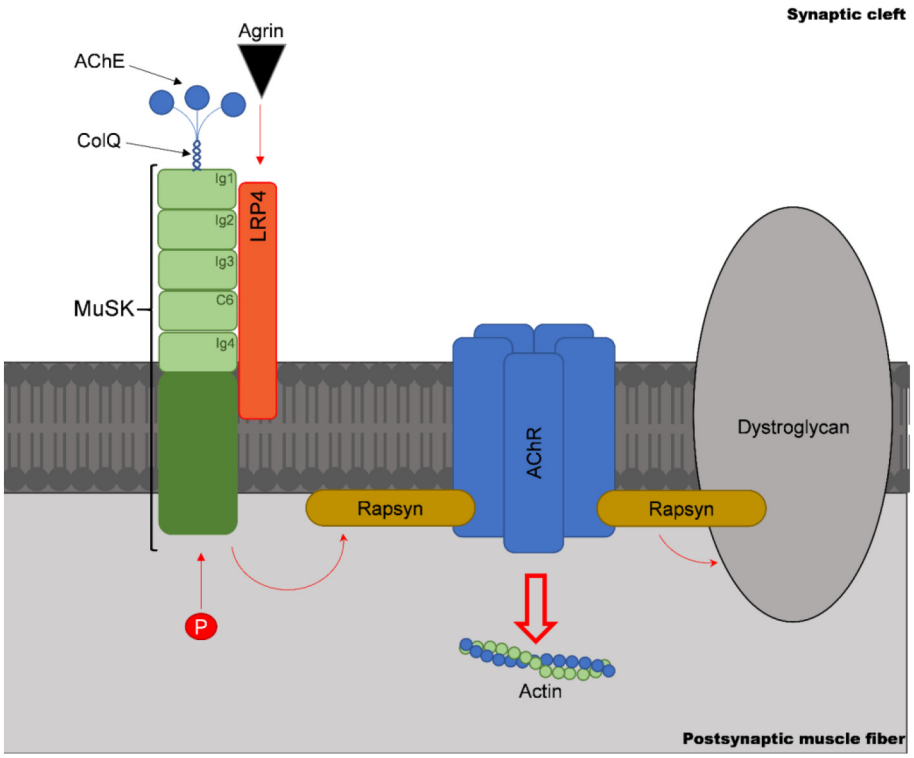

Fig. 3. Schematic representation of part of the post-synaptic apparatus involved in AChRs clustering and target proteins in Myasthenia Gravis. Agrin (released by the presynaptic neuron) binds to LRP4, initiating a cascade of events, by activating MuSK (through phosphorylation). MuSK binds to rapsyn, the protein responsible for anchoring AChR and dystroglycan. Through this process, a high concentration of AChRs is achieved within the NMJ, allowing the action potential to flow from neuron to muscle fibre. Acetylcholinesterase (AChE), the enzyme responsible for the cleavage of ACh into choline and acetate, is essential to remove the excess of acetylcholine and to stop the event. Some of these proteins are targets for antibodies associated with myasthenia gravis (Campanari et al., 2016; Katirji et al., 2013; Huh and Fuhrer, 2002; Otsuka et al., 2015).

antibodies has been found so far, but it is known that these antibodies inhibit MuSK phosphorylation and AChR clustering in vitro (Gasperi et al., 2014). AChR antibodies against titin have been identified in around 20–30% of MG patients, manly those having thymoma-associated disease or late-onset MG (Zisimopoulou et al., 2013; Szczudlik et al., 2014). Titin is located intracellularly and is crucial for muscle contractility (Powers et al., 2014). A more severe thymoma MG and late-onset MG usually reveal titin and ryanodine receptor (RyR) antibodies (Zisimopoulou et al., 2013). Besides that, antibodies against titin are known to be a specific marker of thymoma in patients with MG, where their symptoms appear before 50 years old (Zisimopoulou et al., 2013; Szczudlik et al., 2014). In 10–20% of MG patients, it is also possible to observe antibodies against the α-subunit of the voltage-gated K^+ channel Kv1.4 in skeletal muscle (Suzuki et al., 2009; Romi et al., 2012). Kv1.4 channels are widely present in the Central Nervous System, as well as in the endocardium. In MG disease, Kv1.4 antibodies seem to be cross-reactive with these voltage-gated K^+ channels in the heart muscle, but this antibody binding to Kv1.4 in MG has not been proven in vivo (Suzuki et al., 2009; Romi et al., 2012). Antibodies against ryanodine receptor (RyR) are observed in 70% of AChR-MG patients with thymoma and in 14% of patients with late-onset AChR-MG (Skeie et al., 2003). The RyR antibodies are of IgG1 and IgG3 subclasses, and present the capacity to activate complement protein signalling pathway. The RyR - Ca^{2+} channel in sarcoplasmic reticulum is responsible for depolarization of the sarcolemma, and also acts in muscle contraction through the release of calcium from sarcolemma into the cytoplasm. Although the function of the pathogenic role of anti-RyR antibodies has not been understood yet, its presence is associated with severe MG (Romi et al., 2005).

Collagen Q (COLQ, only found in the NMJ) is a protein that concentrates and anchors acetylcholinesterase (AChE) at the NMJ (Fig. 3), being exclusively present at the extracellular matrix is being therefore a potential target to antibodies. Indeed, antibodies against collagen Q were found in the serum of 3% of MG patients, and some of these patients without other detected antibodies (Zoltowska Katarzyna et al., 2015). These antibodies against collagen Q can be pathogenic, since it was reported that the mutations in *COLQ* were responsible for a form of congenital myasthenic syndromes (Donger et al., 1998). However, the possibility of interference with anticholinesterase therapies still has to be proven (Zisimopoulou et al., 2013). Cortactin (responsible for actin assembly) is a cytosolic actin-binding protein present in the skeletal muscle, being a signalling protein responsible for the AChR clustering mediated by agrin-MuSK complex (Gallardo et al., 2014). Cortactin was found in about 20% of patients with MG but without MuSK or AChR antibodies, as well as in about 5% of patients with MG also with AChR antibodies. Its presence was also found in patients with diverse autoimmune disorders (Zisimopoulou et al., 2013).

3. MG subgroups

Patients suffering from MG can be stratified into subgroups, taking into account the clinical presentation of the disease and its immunopathogenic biomarkers. The criteria for these subgroups are based on the clinical symptoms, group of muscles affected and age of onset, thymic pathology, as well as autoantibodies.

3.1. Ocular MG form

In 15% of patients with MG, ocular symptoms are the only ones that can be detected during the course of the disease. Patients with purely ocular form can develop generalized MG (gMG), especially in the first 2 years upon diagnosis. One-half of these patients with ocular MG has detectable anti-AChR antibodies, but the other half has antibodies only detectable by cell-based assays, thereby not identified in the classical assay.

3.2. Generalized MG form

Patients with early-onset have the first symptoms around the age of 50 or even before, with AChR antibodies in their serum. The histology of thymus in this group is mostly follicular hyperplasia (mainly in women). When the disease onset is stabilized, this group responds well to thymectomy. The age limit of patients suffering from MG for such procedure is however an issue, being thymectomy more beneficial to

younger than to patients older than 50 years (Berrih-Aknin and Le Panse, 2019). Early-onset MG is associated with autoimmune risk genes localized in the major MHC, such as HLA-DR3 and HLA−B8, among other (Renton et al., 2015). Late onset MG form is more common in 50 years old man or older, presenting serum anti-AChR antibodies, and thymic dysfunction. Most of patients show generalized and severe symptoms, being bulbar involvement the most common (Romi et al., 2007). A percentage of about 10 to 15% of all patients with MG show a thymoma-associated MG, which is a paraneoplastic disease. Diagnosed patients possessed anti-AChR antibodies and generalized form of disease (Marx et al., 2013). Besides, in those patients other autoantibodies (anti-ryanodine antibodies, anti-titin antibodies or anti-striated muscle antibodies) are frequently found and, consequently, their detection enables a more precise diagnosis of a thymoma (Suzuki et al., 2011).

Usually, the presence of anti-MuSK antibodies is related to more severe and generalized muscle weakness and atrophy with a notable involvement of cranial and bulbar muscles, mostly among women. In addition, respiratory weakness is most likely in anti-MuSK-MG compared to other subtypes (Evoli et al., 2003). This subgroup does not respond to thymectomy. In this subgroup, HLA association with HLA-DQ5 exists, which is not seen in any other MG subgroups (Bartoccioni et al., 2009; Leite et al., 2005).

3.3. LRP4-associated MG

LRP4 antibodies were discovered in a subgroup of patients which were seronegative for AChR and MuSK antibodies, presenting an ocular or generalized mild MG, more frequently found in women (Higuchi et al., 2011).

3.4. Seronegative MG

Some patients present low-affinity antibodies or even low concentration of antibodies to AChR, MuSK, as well as LRP4 antigen targets, which are not observed in classical immune assays (Leite et al., 2008). These patients can also contain serum antibodies against yet undefined antigens in the NMJ (Cossins et al., 2012).

4. Diagnosis

Together with the identified symptoms of MG (diplopia, ptosis, bulbar disorders, weakness and fatigue), the clinical presentation of the disease is instrumental for a confident diagnosis. However, because of the variability of pathophysiology of MG, in some cases other exams (electrophysiological and immunological tests) are required to complete a successful diagnosis (Meriggioli and Sanders, 2004). Immunological assays for antibodies detection are currently needed to properly diagnose the MG form for each patient. In patients with anti-AChR^{+} antibodies and with a generalized form of MG, CT scan is mandatory to verify the presence/absence of a thymoma.

Diagnostic tests are usually run to confirm the clinical diagnosis, and may include the tensilon test, the ice pack test, the Cogan lid twitch test, the electrophysiological testing and the single-fibre electromyography.

The edrophonium chloride (tensilon) test makes use of a short-acting, reversible acetylcholinesterase inhibitor (edrophonium chloride) with the purpose to inhibit the breakdown of acetylcholine and increase its concentration at the NMJ (Pasnoor et al., 2018). With the increase of the neurotransmitter at the NMJ, it will bind to the postsynaptic receptors causing changes in the ion channels and generating action potential. Although, this is a simple test, reliable, and safe with no need to be performed in the clinical settings (Im et al., 2018), is associated with some shortcomings and can be associated with gastrointestinal, cardiac and pulmonary complications, thus patients should be given a close monitoring. The parameters commonly used by the clinicians are the degree of ptosis in each eye and the palpebral fissure aperture before and after administration of the acetylcholinesterase inhibitor. The test is positive in the presence of the opening of a completely ptotic eye or of a significant increase in the palpebral fissure aperture. However, if the patient does not show ptosis, the tests fail which could lead to a misdiagnosis.

The ice pack test is less sensitive than the tensilon test, but it is commonly used in elderly and in patients that show medical instability to be subject to tensilon test (Pasnoor et al., 2018). It is based on the placing for 1–2 min onto the ptotic eyes of a cold ice pack. Acetylcholinesterase is inhibited by the cold temperature, increasing the availability of the neurotransmitter, improving the neurotransmission and therefore the ptosis (Kee et al., 2019).

In the Cogan lid twitch test, the patient is asked to look down for 10–15 s followed by looking back to the starting position. There is a brief overshooting of the ptotic eyelid before returning to its normal ptotic position. The sensitivity and specificity of this test is low as lid twitch can be the result of other conditions limiting its capacity for diagnosis of MG (Van Stavern et al., 2007).

Repetitive stimulation is the most commonly used electrodiagnostic testing of NM transmission aims to demonstrate the defect by a decremental response of the compound muscle action potentials (CMAP) to slow 2–3 Hz motor repetitive nerve stimulation (Pasnoor et al., 2018). The result is positive if progressively fewer muscle fibres respond to nerve stimulation (Cherian et al., 2013).

The single-fibre electromyography (SFEMG), the most sensitive test seronegative myasthenia gravis diagnosis (Stålberg et al., 1976; Rakocevic et al., 2017), allows the measurement of action potentials from individual muscle fibres (Cherian et al., 2013). Upon consecutive neuronal discharges, variations in the timing of single-fibre action potentials produce the “jitter phenomenon”, which was attributed to variations in the time-point at which muscle action potentials, at motor end-plate, are initiated. SFEMG demonstrated to be useful in detecting the activity-dependent decrease in muscle fibre propagation velocity used in the assessment of muscle fatigue, and the recovery of function, both useful in MG diagnosis. (Pasnoor et al., 2018; Rakocevic et al., 2017). In stimulation jitter studies, usually motor axons are activated by electrical stimulation using a needle electrode inserted near the motor point within the muscle. Stimulation is delivered as pulses (0.05 ms or less duration; 2–3 Hz), with progressive increase in the stimulus intensity, in 0.1 mA steps until small muscle twitches are seen, usually at < 10 mA stimulus intensity. The recording of spikes is achieved by an electrode that is inserted into the twitching part of the muscle. Stimulation jitter studies, are useful in patients that have difficulties in maintaining constant muscle voluntary activation; such as in movement disorders; in too young children; or when the control of firing rate is desirable, for instance to distinguish between pre- and postsynaptic abnormalities (Rakocevic et al., 2017; Sanders et al., 2019). This technique can be used in various muscles, and is very useful to diagnose muscle weakness even at initial stages.

5. Classical therapeutic strategies

As MG disease is characterized by heterogeneity of presentation and severity of symptoms in patients, the choice of its treatment should be based on the subgroup. The classical treatments comprehend the use of molecules for the management of symptoms and also drugs directing to the auto-immune mechanisms of MG, which can encompass short or long-term management of the disease. One of a first-line treatment of MG is the inhibition of acetylcholinesterase at the NMJ, which increases the half-life of acetylcholine and optimizes its capacity to interact with AChR (Sanders et al., 2016). Pyridostigmine 60 mg is the most commonly used drug in the treatment of MG disease, for which the daily intake should be divided during the course of the day (for example, every 6 h). In restricted cases, especially in patients who have dysphagia and other muscle weakness that can compromise their daily-routine, the ingestion of drug precedes meals. It was shown that

patients with anti-MuSK antibodies have a poor response to cholinesterase inhibitors, proving that this strategy is not always recommended (Guptill et al., 2011). Most of MG patients with early or late-onset, thymoma and MuSK$^+$ require immunosuppressive therapy in order to avoid the production of autoantibody and also its effects. It should be noted that in LRP4$^+$ MG, the disease is relatively mild and it does not always required immunosuppression. On the other hand, for ocular MG form, immunosuppression can improve symptoms (ptosis and diplopia) and may avoid generalized weakness (Kerty et al., 2014). Corticosteroids are recognized as the first immunosuppressive therapy widely used in MG. As steroids reduce inflammation and down-regulate the activity of the immune system, these compounds have been used as adjuvants in patients that do not achieve positive therapeutic outcomes with pyridostigmine alone. There is no consensus on the ideal treatment for MG (Dalakas, 2019). Some studies report the use of corticosteroids starting with a high dose of 0.75–1.00 mg/kg daily of prednisone or prednisolone until reaching the expected result and then consecutive low doses are administered to find the lowest dose without the patient revealing any symptoms (Pascuzzi et al., 1984). It is believed that a patient can remain with low doses over several years. Other studies recommend starting with low doses with gradual escalation to limit the risk of side-effects. The corticosteroid therapy should the closely monitored as aggressive therapy with prednisolone plus azathioprine was shown to induce remission in a high percentage of patients with generalized MG and long-term administration of corticosteroids increase the risk of glucose intolerance, gaining of excess bodyweight, of hypertension, and of development of osteoporosis (Gilhus et al., 2016). Non-steroid immunosuppressive agents can be combined with corticosteroids, known as steroid-sparing immunosuppressants. Azathioprine - a purine analogue - inhibits nucleic acid synthesis, interfering with T and B cells proliferation. With a suggested dose of 2–3 mg/kg day, this therapy is a first-line of treatment, being effective in almost all patients with MG. However, it shows some disadvantages, such as delayed effect (usually seen after 6–15 months), as well as the low thiopurine methyltransferase activity, with risk of toxic effects. Thus, thiopurine methyltransferase phenotype can be tested even before the start of treatment (Witte et al., 1984). Mycophenolate mofetil is a prodrug known to selectively constrain the synthesis of guanosine nucleotide in activated T and B cells (Souto and Muller, 2008; Sanders et al., 2008). If the initial immunosuppressive therapy fails, most of the guidelines propose this drug for mild to moderate MG, with a clinical response time of about 11 weeks (Sanders et al., 2008). But there are still some concerns related to the increasing risk of lymphoproliferative disease (Vernino et al., 2005; Dubal et al., 2009). The first immunosuppressant drug that have shown to be efficient in the treatment of generalized MG and thymoma-associated MG was cyclosporine, which is involved with calcineurin signalling, stops cytokine secretion (interleukin-2 and interferon-γ), as well as in T-helper cell activation (Tindall et al., 1987). The initial dose of cyclosporine is 3 mg/kg/day, usually split in 2 intakes. There are also other antibiotics similar to cyclosporine in its biological activity, such as tacrolimus, which is a macrolide antibiotic also used in combination with corticosteroids (Nagane et al., 2005). Methotrexate is a folate antimetabolite responsible for the inhibition of dihydrofolate reductase thereby exhibiting immunomodulatory effect with efficacy similar to azathioprine (Heckmann et al., 2011). Besides their similarities, this drug is used as a second-line treatment and also there are limited data of clinical trials about drug effectiveness (Pasnoor et al., 2016). Cyclophosphamide - an alkylating agent - changes the guanine base of DNA, exhibiting cytotoxic effects and thus stopping B and T cells proliferation. This approach remains a therapeutic option for severe and refractory onset of the disease, but due to the poor tolerability profile of the drug together with the discovery of new alternative immunotherapies, cyclophosphamide is rarely used in MG (De Feo et al., 2002; Drachman et al., 2008).

Intravenous immunoglobulins and plasmapheresis are therapeutic options for acute severe exacerbations in generalized MG, to optimize clinical outcome before surgery and also for short-term immunotherapy as required in incident of MG crisis (Barth et al., 2011; Gajdos et al., 1997). These two therapies are used separately with efficiency improved in patients with severe MG, but occasionally they may be implemented together in order to obtain a maximum response (Gajdos et al., 1997). The combined use of these therapies requires extra care as plasmapheresis will washout IVIG. Plasmapheresis has a faster onset of action being therefore the treatment chosen for MG crisis. A notable response from plasmapheresis of patients with MuSK$^+$ has also been observed (Guptill et al., 2011). A specific system of apheresis, which incorporates the selective immunoadsorption of the anti-AChR antibodies are being studied, showing similar effectiveness and not requiring the replacement of fluid in comparison to plasmapheresis (Kohler et al., 2011). Thymus has a crucial role in MG, justifying this medical approach. Patients with early-onset thymic hyperplasia and patients with thymoma responded well to the treatment (Cea et al., 2013; Gronseth and Barohn, 2000). Anti-AChR antibodies have shown advantages to be used in this group as markers of early-onset or of thymoma present in the serum of the patients. Nevertheless, patients with thymoma have more critical disease and thus the removal of the tumour cannot conduct to total remission (Gronseth and Barohn, 2000). Patients should therefore be stable prior the treatment. To reduce the risk of complications intravenous immunoglobulin or plasmapheresis therapy should be done before surgery (Gajdos et al., 1997).

6. Innovative therapeutic targets

Eculizumab - a humanized monoclonal antibody – specifically binds the complement protein C5 (Fig. 4), thereby not allowing its cleavage into C5a and C5b (Dhillon, 2018; Howard Jr et al., 2017). The complement is exclusively activated by anti-AChR antibodies, thus this monoclonal antibody can be a possible strategy for patients with generalized anti-AChR$^+$ MG who were refractory to other treatments (Silvestri and Wolfe, 2014). Its safety and efficacy have already been demonstrated in a few clinical trials with refractory anti-AChR$^+$ gMG patients (Howard Jr et al., 2017; Howard Jr. et al., 2013). Focusing on the innate immune system, the terminal complement inhibition by eculizumab can thus be a suitable strategy in the management of patients with severe and refractory anti-AChR$^+$ gMG. This humanized monoclonal antibody has already been approved for the treatment of other diseases, such as Paroxysmal Nocturnal Haemoglobinuria (PNH) and Atypical Hemolytic uremic Syndrome (aHUS) (Rother et al., 2007; Wong and Kavanagh, 2015).

Rituximab is another monoclonal antibody against transmembrane-protein CD20 which is present in B lymphocytes (Fig. 4) (Tandan et al., 2017). This transmembrane protein CD20 contributes to activation and differentiation of B cells, depleting them through cell mediated lysis by binding of macrophages or natural killer cells to Fc-receptor, complement activation of the membrane attack complex, as well as induction of cell apoptosis by changing lipid membrane in B cells (Vander Heiden et al., 2017; Tedder and Engel, 1994). Rituximab was firstly used in the treatment of B-cell malignancies (on-Hodgkin's lymphoma), and also in several autoimmune diseases (rheumatoid arthritis, systemic erythematous lupus and also pemphigus vulgaris) (Kosmidis and Dalakas, 2010). Published data and some case series indicate that rituximab shows efficacy in AChR$^+$ and in MuSK$^+$ patients with generalized and severe disease form who do not respond to other treatments (Hain et al., 2006). Its action is nevertheless more effective in MuSK$^+$ MG patients. Immunopathogenesis of the MG needs to be clearly understood in order to discover specific immune targets which are decisive to the development and maintenance of the disease, allowing a more focused treatment and less adverse side effects.

Targeting T-cell co-stimulatory, and also co-inhibitory pathways, has become a crucial therapeutic approach for immunotherapy of autoimmune diseases, transplantation and for cancer (Felix et al., 2010).

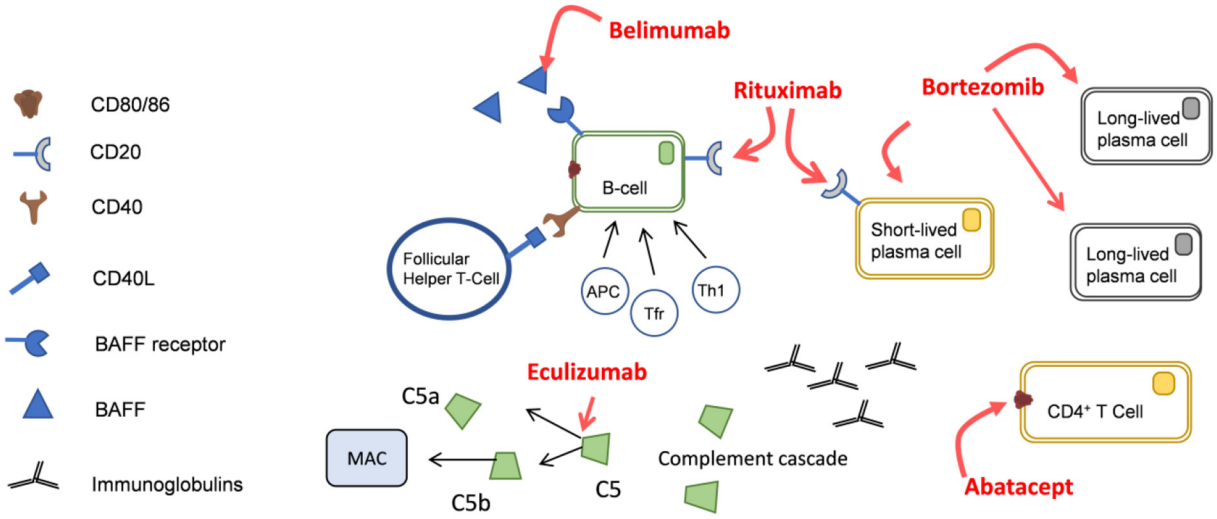

Fig. 4. Myasthenia gravis therapies and their targets (adapted from (Beecher et al., 2019); see text for details).

T-cells are responsive because they can recognize a specific complex of peptide-major histocompatibility complex by the T-cell receptor in association with co-signalling pathways that stimulate or constrain T-cell activation, promoting T-cell response (Jung and Choi, 2013). CD28 - T cell-specific surface glycoprotein CD28 - is a co-stimulatory molecule for $CD4^+$ helper T cells and it can bind the CD80 and CD86 ligands expressed on antigen presenting cells (APCs). The co-stimulation of CD28 increases the responses of T-cell in naïve cells and the production of cytokine (mainly IL-2), which binds to CD25 on T cells thereby promoting proliferation. CTLA-4 - cytotoxic T-lymphocyte protein 4 - is a co-inhibitory molecule expressed on $CD4^+$ T cells after activation, also binding CD80 and CD86, and is crucial for the down-regulation of antigen-activated immune responses (Chen and Flies, 2013). The use of CTLA-4-Ig (abatacept) - an immunoglobulin fusion protein - can block this pathway, through its connection to CD80/CD86 thereby blocking the activation (CD28) and the inhibitory signals (CTLA-4). Abatacept has been clinically tested, showing to be effective and safe, being currently used in the treatment of rheumatoid arthritis (Keating, 2013). But focusing on the type of T cell response, the co-stimulation blockade with abatacept has different effects. MG associated to AChR was shown to be primarily mediated by the responses of T helper (Th) 1 T-cells (Th1), although the involvement of T cells production of IL-17 is also present (Schaffert et al., 2015; Gradolatto et al., 2014). Other reports describe that the expression of CTLA-4 on T cells can be altered in MG. Besides, an association signal at the CTLA-4 gene in a genome-wide association study in patients with AChR-positive MG was also reported, demonstrating that aberrant cellular mechanisms involving CTLA-4 can predispose to MG and therefore therapies targeting this pathway should be considered for the treatment of this disease (Renton et al., 2015). After the activation, T cells manifest another co-stimulatory molecule - the inducible T cell co-stimulator protein (ICOS) - that binds to ICOS-ligand on APCs, regulating the activation of T and B cells (Nurieva, 2005). This was proposed as a useful strategy for MG, once the inhibition of ICOS can enhance autoimmunity in diseases where antibodies are produced.

OX40 is known to be another co-stimulatory molecule responsible for the activation of T cell, by binding to OX40L on the APC and improves cytokine production, proliferation, as well as the survival of T cells (Ishii et al., 2010) (Fig. 5). It is well documented that OX40-OX40L pathway commands the suppression of Tregs, thus the targeting of this pathway is worth to further investigation (Croft et al., 2009). The lack of functional Tregs can explain the autoimmune diseases, besides a subset of T cells ($CD4^+CD25^+$ Tregs) allows the maintenance of immune homeostasis against self-antigens (Sakaguchi, 2004). Tregs have been implicated in preventing autoantibody production, and their dysfunction or deficiencies have been reported in MG, which suggest them as a potential target (Masuda et al., 2010). CD40 is known to be expressed on APCs (macrophages and dendritic cells) as well as on B cells (Zhang et al., 2013). CD154 or CD40L - the ligand for CD40 - is expressed on T cells that are activated, thus targeting CD154 can diminish B cells activation and thus diminish the AChR-specific humoral response (Im et al., 2001). Th1 and also Th17 cells were shown to be involved, being indispensable for the responses of AChR-specific B-cell in MG combined with anti-AChR antibodies (Berrih-Aknin and Le Panse, 2014). The interaction between T-cells and antibody-producing B cells is not yet well-understood in MuSK-positive MG. It is possible that B cell depletion can be due to the elimination of anti-MuSK antibody-producing plasmablasts, and thus targeting CD40 can be adequate in MuSK MG as CD40 signalling for B-cell differentiation in plasmablasts (Zhang et al., 2013). Besides, T follicular helper (TFH) cells contribute with B cells which help during an autoimmune response, making them a developing target, once their dysfunction are greatly involved in this autoimmunity pathway (Winkler and Waisman, 2014). Targeting B cells present advantages through the reduction of antibody-producing cells, but also through the modulation of other B-cell functions (antigen presentation and cytokine production). A subset of B cells - regulatory B cells or B10 cells - have been identified based on their production of IL-10 and on suppressing B-cell responses (Tedder, 2015). It is known that MG patients have reduced B cells in circulation, being correlated with the severity of the disease, while patients responding to B cell depletion with rituximab present a faster re-population of B10 cells (Sun et al., 2014).

BAFF - a survival factor - is crucial for proliferation and differentiation of B cells (Schneider et al., 1999). Studies have shown that BAFF serum levels are increased in patients with $AChR^+$ and $MuSK^+$ MG, being significantly high in patients with active $AChR^+$ (Ragheb and Lisak, 2011; Ragheb et al., 2008). Belimumab - human monoclonal antibody targeting BAFF - binds to a soluble BAFF instead of a membrane-bound BAFF on B cells, thus causing a reduction in activation and differentiation of B-cell in antibody-producing plasma cells (Kao et al., 2014). Bortezomib is an inhibitor of proteasome activity in plasma cells, interrupting in this way the proteolytic pathways and thereby causing the protein accumulation in plasma cells, culminating in cell death. In experimental MG, bortezomib exhibited a reduction of AChR antibodies showing a clinical improvement (Gomez et al., 2011). Because of their interaction with IgG, Fc receptors are involved in the humoral and cellular immune responses, becoming therefore a viable target to treat autoimmune diseases. IgG antibodies interact with

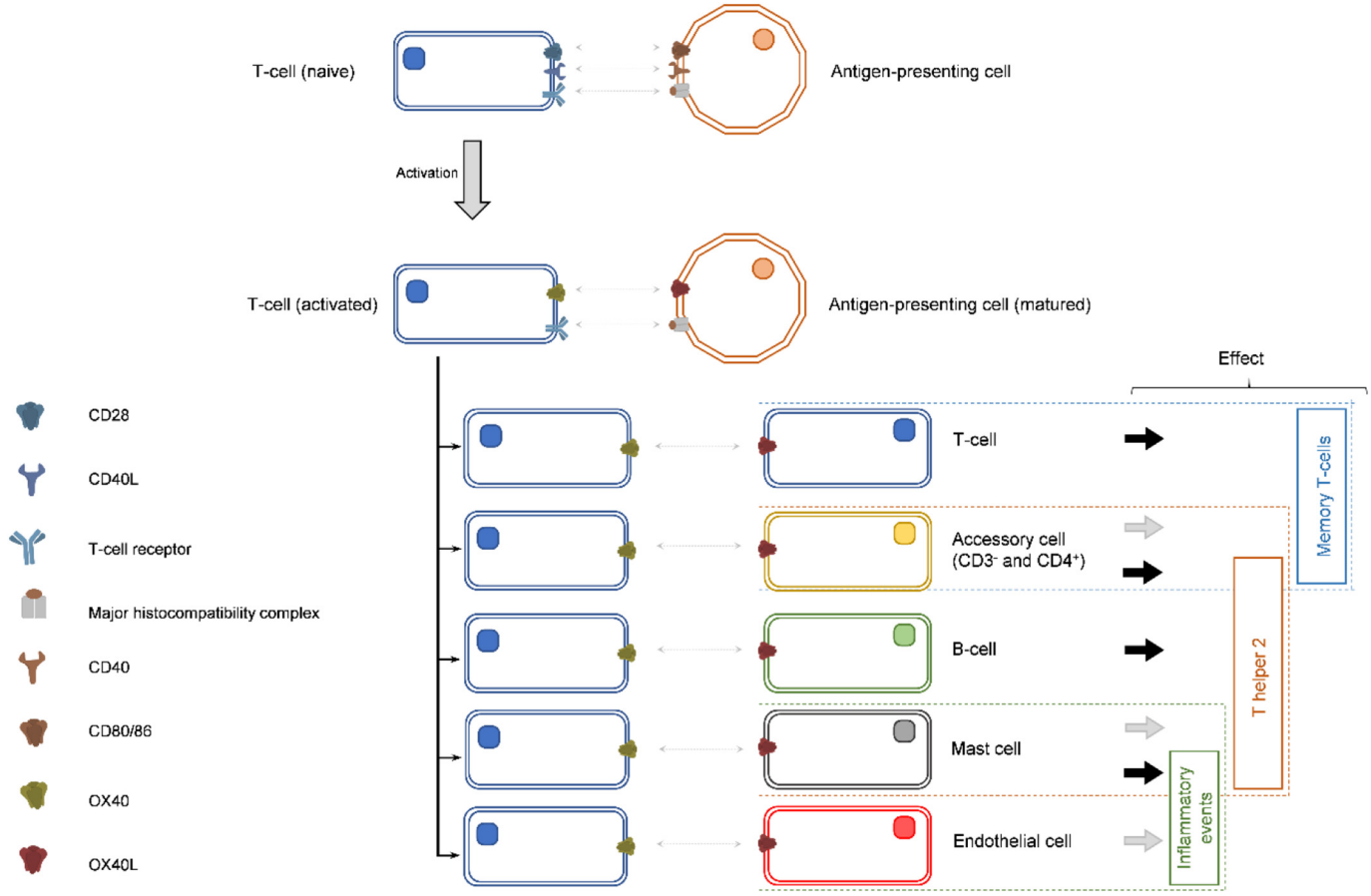

Fig. 5. Ox40/OX40L co-stimulatory interactions. When in the presence of antigen-presenting cells (APC), naive T-cells recognize the APC's and undergo activation. After this period, OX40 expression in T-cells is induced by the T-cell receptor pathway and these cells become able to interact with other cells expressing OX40L. If this second cell-cell interaction is between a T-cell and another T-cell the resulting effect is generation of memory T-cells; if the interaction is with an accessory cell expressing $CD3^+/CD4^-$ or a B-cell the effect observed is the promotion of T helper 2; an inflammatory response is observed if T-cells interact with a mast cell. The possible effects are identified with filled black arrows, but these interactions can also provide the same effect with different interactions (hypothetical effects, indicated by filled grey arrows).

immune system through the Fcγ receptors expressed on immune cells, comprehending B cells. Through the positive and negative regulatory signals, these interactions can modulate the immune response. Positive signals manifest themselves through the FcγRs which have diverse affinities to IgG Fc taking into account the IgG isotype. Negative signals, on the other hand, are shown by the inhibition of FcγRIIb expressed on B cells which, when activated, suppress the activation of B cells (Pincetic et al., 2014). Intravenous immunoglobulins have an influence on Fc receptors, as glycosylation of Fc core can affect the therapeutic effect, improving efficacy in autoimmune disease (Kaneko et al., 2006). Through interaction with Fcγ receptors, IgG Fc domains can explain the effect of IVIg in Mg (Nagelkerke and Kuijpers, 2014). Targeting FcR-IgG interactions can decrease endogenous IgG and become an interesting approach. The design of biospecific ligands targeting the inhibitory FcγRIIb and B-cell receptor is another promising treatment approach (Horton et al., 2011).

Th1 and Th2 cytokines are associated with the pathogenesis of MG, showing differences in each MG subgroup. Targeting Th1 cytokines (IL-12 and tumour necrosis factor-α) have shown positive results in experimental models, however this approach has been difficult to apply in clinic due to safety reasons. Th17 immune reactions are important in MG, as some cytokines (IL-17 and IL-6) are recognized as interesting therapeutic targets. Several human monoclonal antibodies against IL-17 and IL-6 (e.g. brodalumab, ixekizumab, secukinumab) are currently under study. In the MG disease, IL-6 is a promoter of differentiation and proliferation of B-cell and it can also induce the maturation of B-cell in antibody-producing plasma cells (Bao and Cao, 2014). GM-CSF is a cytokine that acts as a hematopoietic growth factor and as immune modulator, interfering with different circulating immune cells. There are some studies in various autoimmune models about its effects on dendritic cell maturation and on the enhancement of Treg function (Bhattacharya et al., 2015). It is known that GM-CSF can enhance the in vitro suppressive function of isolated Tregs from patients with MG, through the up-regulation of FOXP3 expression in these cells (Thiruppathi et al., 2012). In a specific patient with severe MG, the treatment with GM-CSF enhanced FOXP3 expression in Tregs and increased Treg-mediated suppression of T-cell proliferation followed by polyclonal or AChR-specific stimulation (Rowin et al., 2012). However, clinical trials are still needed to verify the safety of GM-CSF to expand its use as a treatment approach.

The immune system tolerance to AChR has been investigated in experimental MG models by administering AChR peptides both by oral and nasal routes (Paas-Rozner et al., 2000; Shi et al., 1998). DNA vaccinations (peptide vaccines) have also been proposed, but there still is a concern with the potential exacerbation of anti-AChR immune response. A recent promising antigen-specific therapeutic strategy is the immunization with AChR cytoplasmic domains (Luo and Lindstrom, 2014). Another therapeutic strategy for refractory MG, i.e. the approach of autologous stem cell transplantation, has already been tried in other autoimmune diseases (Passweg and Tyndall, 2007).

7. Conclusions

The current strategies in Myasthenia Gravis (MG) are based on generalized immunosuppression and on symptomatic treatment, and not on specific targeting of potential antigen-presenting molecules. The study and development of new targeted therapies based on immune pathogenesis of MG can improve quality of life of patients who have side effects associated with chronic uptake of currently used drugs, being an alternative for people who are refractory to those therapies. Indeed, MG is a heterogeneous disease, which implies that future therapeutic approaches should be adapted based on each MG subgroup. In the near future, with growth on innovation in science and technology, more clinical trials are expected to be run with the emergence of new molecules and more efficient therapeutic options.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This research was funded by Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) under the project references M-ERA-NET/0004/2015 (PAIRED) and UID/AGR/04033/2019 (CITAB), co-financed by FEDER, under the Partnership Agreement PT2020.

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