



Review

Myasthenia gravis: A comprehensive review of immune dysregulation and etiological mechanisms



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ABSTRACT

Autoimmune myasthenia gravis (MG) is characterized by muscle weakness caused by antibodies directed against proteins of the neuromuscular junction. The main antigenic target is the acetylcholine receptor (AChR), but the muscle Specific Kinase (MuSK) and the low-density lipoprotein receptor-related protein (LRP4) are also targets. This review summarizes the clinical and biological data available for different subgroups of patients, who are classified according to antigenic target, age of onset, and observed thymic abnormalities, such as follicular hyperplasia or thymoma.

Here, we analyze in detail the role of the thymus in the physiopathology of MG and propose an explanation for the development of the thymic follicular hyperplasia that is commonly observed in young female patients with anti-AChR antibodies. The influence of the pro-inflammatory environment is discussed, particularly the role of TNF- α and Th17-related cytokines, which could explain the escape of thymic T cells from regulation and the chronic inflammation in the MG thymus. Together with this immune dysregulation, active angiogenic processes and the upregulation of chemokines could promote thymic follicular hyperplasia.

MG is a multifactorial disease, and we review the etiological mechanisms that could lead to its onset. Recent global genetic analyses have highlighted potential susceptibility genes. In addition, miRNAs, which play a crucial role in immune function, have been implicated in MG by recent studies. We also discuss the role of sex hormones and the influence of environmental factors, such as the viral hypothesis. This hypothesis is supported by reports that type I interferon and molecules mimicking viral infection can induce thymic changes similar to those observed in MG patients with anti-AChR antibodies.

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1. Introduction

Myasthenia gravis (MG) is characterized by fluctuating muscle weakness and abnormal fatigability. MG is an autoimmune disease caused by the presence of antibodies against components of the muscle membrane at the neuromuscular junction. In most cases, autoantibodies against the acetylcholine receptor (AChR) can be found. Recently, other targets, such as Muscle-Specific Kinase (MuSK) and Lipoprotein-Related Protein 4 (LRP4), have been

described. MG is classified based on the location of the affected muscles (i.e., ocular versus generalized), the age of onset of symptoms, and the autoantibody profile. These criteria are required to optimize the management and treatment of MG patients.

The origin of the autoimmune dysfunction in MG patients is unknown, but thymic abnormalities, defects in immune regulation and sex hormones play major roles in patients with anti-AChR antibodies. Genetic predisposition is also likely to influence the occurrence of the disease.

In this review, we analyze the latest concepts related to the pathophysiology of MG according to the different subgroups of the disease and provide a description of the roles of immunological, genetic, hormonal and environmental factors in the development of this disease.

2. Classification

MG occurs in patients of all ages and both sexes. The incidence ranges from 1.7 to 21.3 per million, and the prevalence is between

Abbreviations: AChR, acetylcholine receptor; AIRE, autoimmune regulator; EAMG, experimental autoimmune myasthenia gravis; ER, estrogen receptor; GC, germinal center; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MG, myasthenia gravis; EO, early-onset; LO, late-onset; MHC, major histocompatibility complex; miRNA, microRNA; LRP4, lipoprotein-related protein 4; MuSK, muscle-specific kinase; PBMC, peripheral blood mononuclear cells; Poly(I:C), polyinosinic-polycytidylic acid; Tconv, conventional T cells; TEC, thymic epithelial cell; TNF, tumor necrosis factor; Treg, regulatory T cells; TLR, toll-like receptor.

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15 and 179 per million inhabitants, depending on the location [1,2]. Studies of large groups of patients show that there is a predominance of female cases (60–70%) before the age of 50 years but not after the age of 50 years.

The clinical presentation, the age of onset, the autoantibody profile, and the thymic pathology can differ among patients and are used to define several subgroups of patients (Table 1). The different antibodies involved in MG are all directed against proteins of the neuromuscular junction (Fig. 1).

2.1. Forms of MG that present with anti-AChR antibodies (AChR-MG)

Acetylcholine receptors (AChRs) are found on the surface of muscle cells, concentrated at the synapse between nerve cells and muscle cells. AChRs are composed of five protein chains (i.e., $2\alpha\beta\epsilon\delta$ for the adult form and $2\alpha\beta\gamma\delta$ for the fetal form) that are arranged into a long tube, which forms a channel that crosses the cell membrane. The α chains have binding sites for acetylcholine on the external side and contain the primary immunogenic region that is recognized by anti-AChR autoantibodies. When acetylcholine binds to these two chains, the shape of the entire receptor changes slightly, opening the channel. This conformational change allows positively charged ions to cross the membrane, generating endplate potentials and leading to muscle contraction. In MG patients with anti-AChR antibodies, the density of AChR at the neuromuscular junction is reduced, which results in decreased endplate potentials. Disease severity is correlated with the loss of AChRs, as measured in muscle biopsies [3], but not with levels of circulating antibodies [4]. Electron microscopic studies of the neuromuscular junction reveal a disruption of the architecture of the post-synaptic region, characterized primarily by a simplification of synaptic folds [5].

A number of mechanisms underlie the reduction of AChRs at the neuromuscular junction in MG patients [6]. The primary mechanism is the destruction of the postsynaptic membrane by complement pathway activation, which leads to the generation of the membrane attack complex [7]. Antigenic modulation and AChR blocking mechanisms have also been described [8]. In some cases, blocking antibodies play a major role. For example, in neonatal myasthenia gravis, maternal antibodies recognize the fetal form of the AChR and inhibit neuromuscular transmission in the baby, leading to a transient MG at birth. In this case, the blocking effects appear to trigger neonatal MG and are correlated with the severity of the disease in the child [9].

Muscle is not a passive target of the autoimmune attack that occurs in MG patients. The loss of AChRs at the neuromuscular junction is compensated by the active synthesis of different AChR subunits [10,11]. Thus, the level of AChR expression at the muscle

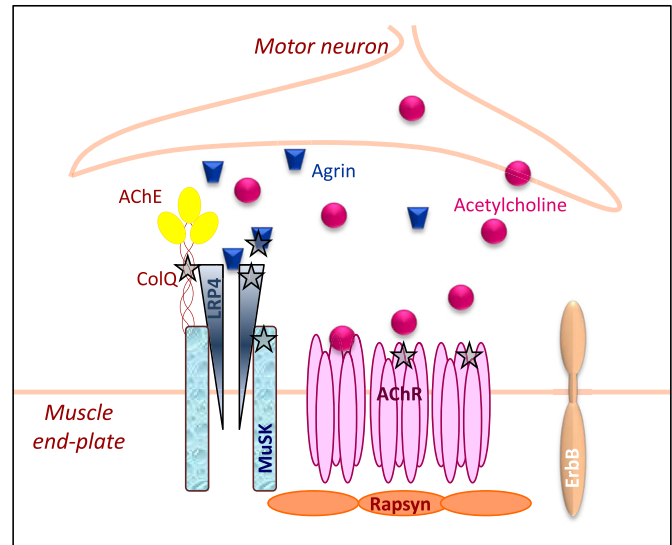


Fig. 1. Simplified schematic diagram of the neuromuscular junction. The main components of the neuromuscular junction that are involved in MG are illustrated. AChE: acetylcholine esterase; AChR: acetylcholine receptor; ColQ: collagen-tail subunit of AChE; ErbB: receptors for neuregulins; LRP4: lipoprotein related protein 4; MuSK: muscle-specific kinase. Agrin released from motor neuron terminals binds to the Lrp4-MuSK complex, inducing MuSK phosphorylation and activating signaling pathways that lead to AChR clustering. The grey stars represent the known antigenic targets in MG.

endplate is the result of both its degradation by autoantibodies and its synthesis via compensatory mechanisms.

Several clinical subgroups of MG patients with anti-AChR antibodies have been identified, as described below.

2.1.1. Ocular form

This form is diagnosed when symptoms of the disease are limited to ocular symptoms for at least 2 years. Approximately 15% of all AChR-MG patients are affected by this form of the disease. In half of these patients, anti-AChR antibodies are detectable in the classical immunoprecipitation assay [12]. In the remaining patients, most of them have anti-AChR antibodies only detectable in the cell-based assay, in which the AChR is clustered [13].

2.1.2. Generalized form

Patients with a generalized form of MG can be divided into three subgroups according to the age of onset.

- **Early-onset form (EOMG) (age of onset <50 years).** Most early-onset MG (EOMG) patients present with a high level of anti-

Table 1
Classification of MG patients according to the nature of the observed autoantibodies.

	Autoantibody target			
	Solubilized AChR	Clustered AChR	MuSK	LRP4
Percentage of patients	85%	~5%	~4%	~2%
Targeted populations	Early onset: F > M Late onset: F = M		Young females	Young females
Severity grade	All severity grades: Ocular and generalized forms		Severe forms	Mild forms
Pathogenicity	<i>In vitro</i> <i>In vivo</i>	<i>In vitro</i>	<i>In vitro</i> <i>In vivo</i>	<i>In vitro</i>
Isotypes	IgG1, IgG3	IgG1	IgG4	IgG1
Role of complement	Yes	Likely	No	Likely
Thymic pathology	EOMG: follicular hyperplasia LOMG: thymoma	Mild follicular hyperplasia	No	?
Correlation of Ab titer with disease grade	No	?	Yes	?

AChR antibodies and thymic follicular hyperplasia that is characterized by ectopic germinal centers (GCs) [14]. Sex hormones may play a role in this form of the disease, as more than 80% of patients with follicular hyperplasia are women [15]. Patients in this subgroup may have other autoantibodies and can develop other autoimmune diseases, such as thyroiditis [16].

- **Late-onset form (LOMG) (age of onset >50 years).** This subgroup is frequently associated with the presence of a thymoma. The presence of autoantibodies against striated muscle proteins, such as ryanodine or titin is common in late-onset patients [17]. This form of MG is usually generalized and severe, with bulbar signs and frequent severe respiratory crises [18].
- **Very late-onset form (age of onset >60 years).** In recent decades, a form of MG that appears after 60 years of age has been described. This form predominantly affects males, and it is distinct from LOMG, as patients do not present with thymoma [19]. In a study conducted in Italy between 1987 and 2007, the incidence of patients older than 50 years without thymoma tripled after 1990 [20]. Because the incidence of the EOMG did not increase in parallel, this effect is probably not related to improved diagnosis. The increased incidence of this very late form of the disease may be due to increased longevity within the population. Because signs of autoimmunity increase with age, elderly individuals are more likely to develop an autoimmune disorder such as MG. The accumulation of predisposing environmental factors over time may also explain these epidemiological trends.

2.2. The form of MG that presents with anti-MuSK antibodies (MuSK-MG)

The MuSK protein plays a major role in the development of the neuromuscular junction [21] and is essential for the clustering of the AChR, as no aggregates of AChRs were observed in its absence [22]. Approximately 40% of patients with generalized symptoms and without anti-AChR antibodies have anti-MuSK antibodies. Most of these patients are young females [23]. These patients typically have severe clinical symptoms with involvement of the facial, bulbar and respiratory muscles, but they rarely have ocular symptoms. Muscle atrophy is common in these patients [24]. In general, no thymic pathology is observed in this subgroup of patients [24,25]. In contrast to AChR-MG, most anti-MuSK antibodies are of the immunoglobulin (Ig)G4 isotype and do not bind complement. The pathogenicity of the IgG4 isotype was confirmed *in vivo*, as anti-MuSK IgG4 but not IgG1–3 induced muscle weakness in mice [26] (Table 1). In addition, postsynaptic AChR clusters are severely fragmented, with a subsequent reduction of the presynaptic nerve terminal area that is correlated with the disease grade in the experimental autoimmune MG (EAMG) [27]. Thus, the mechanism of action of anti-MuSK antibodies appears to be very different from that of anti-AChR antibodies.

2.3. The form of MG that presents with anti-LRP4 antibodies (LRP4-MG)

The LRP4 protein belongs to a family of proteins that has been recently identified as the receptor for the neural agrin that can activate MuSK [28]. Its role in the formation of endplates was demonstrated in mice with a mutated form of LRP4; these mice die at birth because of respiratory distress, as do mice with mutated forms of MuSK or agrin [28]. Approximately 20% of patients with a generalized form of MG without anti-AChR or -MuSK antibodies have anti-LRP4 antibodies. This form has been recently described [29,30]. The profile of patients with this type of anti-LRP4 antibodies is reported in Table 1 and fully described by Tsartos' team in this issue [31].

2.4. Seronegative MG

Seronegative patients are heterogeneous and may have a pure ocular or a generalized form of MG [32]. Some of these patients appear to have anti-AChR antibodies that are not detectable by the classical immunoprecipitation assay, as their autoantibodies only recognize AChR in its native configuration. This was demonstrated via a cell based-assay (CBA) that used genetically modified cells expressing the different subunits of muscle AChR together with rapsyn, a cytoplasmic protein that is required for the stabilization of AChR. Up to 60% of seronegative MG patients have antibodies against the clustered AChR in this cell-based assay. These patients are similar to patients with anti-AChR with respect to their clinical presentation, their response to treatment, and their thymic abnormalities [13]. In these patients, the anti-AChR antibodies are predominantly of the IgG1 isotype and can therefore activate complement (Table 1).

Antibodies against other molecules in the endplate have also been investigated. Some patients have antibodies against collagen Q or agrin [33]. Fig. 1 depicts the neuromuscular junction and the different targets of the autoimmune response in MG patients.

3. The role of the thymus in MG

The thymus is essential for T-cell differentiation and for the establishment of central tolerance. Interactions between thymic stromal cells expressing self-antigens and developing thymocytes lead to the elimination of autoreactive T cells. The self-tolerant T cells continue their differentiation before being exported to the periphery. Thymic stromal cells include epithelial cells [34], mesenchymal cells [35], and a few myoid cells [36].

Under physiological conditions, most thymic cells are thymocytes and stromal cells. The number of B cells is very small. In the majority of MG patients (i.e., in AChR-MG patients), the thymus exhibits structural and functional changes that are characterized by the presence of a tumor (i.e., thymoma) or by the development of germinal centers (GCs) containing a large number of B cells (i.e., follicular hyperplasia) [37]. In EOMG, follicular hyperplasia is very common, while in the LOMG, thymomas are frequently observed. These morphological changes of the thymus are primarily associated with the AChR-MG.

3.1. Thymoma

Thymomas are caused by the abnormal development of epithelial cells. The most recent WHO classification of thymomas is based on the nature of the cortical or medullary epithelial cells involved in the tumor: Type A (i.e., medullary thymoma), type B1 or B2 (i.e., mainly or entirely cortical thymoma, respectively), type AB (i.e., mixed medullary and cortical thymoma) or type B3 (i.e., atypical thymoma, squamoid thymoma and well-differentiated thymic carcinoma) [38]. Approximately 10–20% of generalized AChR-MG patients have a thymoma, usually type B1 or B2. These patients are typically older than 40 years of age. There is a strong link between thymoma development and autoimmune mechanisms. A recent study that included 302 thymoma patients demonstrated that MG was observed in 55% of patients, and other autoimmune syndromes were observed in 39% of patients [39]. The medullary area in the thymoma is usually limited. However, thymopoiesis is maintained and a large number of naive cells are exported into the blood. Because the thymic medulla is the site of negative selection, we can assume that thymic cells exported to the periphery from a thymoma may include autoreactive T cells that have not been effectively deleted. In addition, several molecular components that are important for tolerance are deficient in

thymomas, including the autoimmunity regulator factor (AIRE), the master gene for regulatory T (Treg) cell function (i.e., FoxP3) and the major histocompatibility complex (MHC) class II antigens (reviewed in Ref. [40]). This increased self-reactivity may also explain the frequent presence of antibodies against titin, ryanodine and cytokines in the serum of patients with thymoma [40,41]. Indeed, patients with thymoma are characterized by the presence of anti-interferon (IFN) type I antibodies [41]. A recent study demonstrated that thymomas from MG patients express high levels of type I IFNs, such as IFN- α 2, - α 8, - ω and - β . The overexpression of these cytokines was specifically observed in thymomas from MG patients [42].

3.2. Thymic follicular hyperplasia

3.2.1. Ectopic germinal centers (GCs)

Ectopic GCs developing in the thymus are primarily associated with AChR-MG but not MuSK-MG [25,43]. Patients with thymic follicular hyperplasia typically display elevated serum AChR antibody titers [44]. In these patients, the thymus is one site of anti-AChR production [45–47]. It is worth noting that B cells from the MG thymus are already activated and do not require additional activation to produce anti-AChR antibodies [45]. The specific activity of the spontaneously produced IgG (i.e., anti-AChR/total IgG) is much higher in the thymus than in the bone marrow, the peripheral blood, or the lymph nodes [48,49]. In addition, thymic tissue or dissociated thymic cells from the AChR-MG patients can induce the production of antibodies against AChR in immunodeficient mice and the loss of AChR at the muscle endplates [50,51], demonstrating that the MG thymus contains B cells producing anti-AChR antibodies.

In MG patients, thymic GCs are sensitive to anti-inflammatory corticosteroid therapy (Fig. 2). GCs are occasionally observed in the thymuses of healthy individuals, but their number and size are very small compared to MG patients who have not been treated with corticosteroids [52,53]. Similar GCs have also been described in inflamed tissues in patients with other autoimmune diseases, such as the salivary glands in patients with Sjogren's disease, the joints in patients with rheumatoid arthritis, and the meninges in patients with secondary progressive multiple sclerosis [53,54]. Thus, the thymus appears to be the inflamed tissue in AChR-MG patients, just as the thyroid is the inflamed tissue in thyroiditis and the salivary glands are the inflamed tissue in Sjogren's disease [55].

3.2.2. Neo-angiogenesis and chemokines in thymic follicular hyperplasia

Follicular hyperplasia in the thymus of MG patients is characterized by active neo-angiogenesis [14]. These new vessels,

including high endothelial venules (HEVs) and lymphatic vessels, play an important role in the recruitment of peripheral cells via interaction with chemokines, which contribute to the abnormal development of thymic GCs [14,52–54]. The mechanism by which ectopic HEVs develop in inflamed tissues remains unknown. In experimentally induced organogenesis, lymphotoxin plays a critical role in HEV formation [56,57]. However, when MG and non-MG patients were compared using thymic transcriptome analysis [58] or real-time PCR [53], no significant differences in lymphotoxin- α and - β mRNA expression were observed.

The molecules responsible for the migration of B cells into inflamed tissues are primarily the chemokines CXCL13, CCL21, and SDF-1/CXCL12, which also participate in the generation of GCs under both physiological and pathophysiological conditions [55]. DNA microarray analysis revealed a significant overexpression of CXCL13 and CCL21 in the thymus of MG patients [58]. CXCL13, which is also known as B-lymphocyte chemoattractant (BLC) [59], was produced not only by B cells and follicular dendritic cells, but also by thymic epithelial cells (TECs). TECs from MG patients overproduce this chemokine [52]. The population of cells expressing CXCR5, the receptor for CXCL13, which plays a central role in B cell interactions, is increased in the peripheral blood of MG patients [60,61]. Interestingly, CCL21 overexpression in the MG thymus is primarily due to the lymphatic vessels, and CCL21 was shown to attract naive B cells in the human and murine systems [14]. SDF-1 was found at the lumen of HEVs, and many antigen presenting cells, including B cells, were identified around these vessels, suggesting that HEV development and the engagement of SDF-1 contribute to MG pathology by recruiting peripheral B cells and antigen presenting cells to the MG thymus [53].

Altogether, these findings demonstrate that the chemokines CXCL13, CCL21, and SDF-1 are involved in the attraction of peripheral B cells to the MG thymus via active angiogenesis. In this context, it was recently observed that the chemokines CCL17 and CCL22 were overexpressed in MG thymuses when compared to normal thymuses. These chemokines were mainly detected around medullary Hassall's corpuscles, and they were induced by toll-like receptor (TLR)4 activation, driving DC recruitment into the MG thymus [62].

3.2.3. Can inflammation induce follicular hyperplasia?

A transcriptome analysis of the hyperplastic thymus and the muscle from MG patients revealed signs of inflammation in the thymus but not in the muscle. Because autoimmune sensitization against the AChR likely develops in the thymus, inflammatory cytokines play a crucial role in MG pathogenesis.

The inflammatory state of the MG thymus was demonstrated by the overexpression of numerous IFN- γ -induced genes [58,63]. A

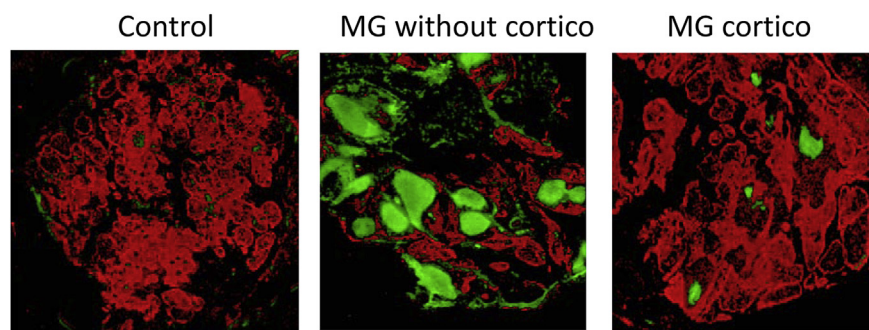


Fig. 2. The MG thymus. These sections are from young women (22–26 years of age). The red staining corresponds to the epithelial network (anti-keratin antibody). The green staining corresponds to the B cells and the follicular dendritic cells (anti-CD21 antibody). Young female MG patients frequently present with a large number of germinal centers, and corticotherapy reduces the number of germinal centers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thorough analysis of the effects of IFN- γ on AChR subunit expression in TEC and thymic myoid cell cultures demonstrated that IFN- γ is a potent inducer of the AChR subunits, particularly AChR- α [63], the subunit that includes the main immunogenic region [64].

Several activation markers that are linked to T-cell function have also been associated with MG. A T cell subset expressing high levels of Fas is strongly enriched in the thymus and participates in the anti-AChR response [65]. These cells accumulate only in the thymus of patients with anti-AChR antibodies [66]. In addition, thymic and peripheral lymphocytes from MG patients exhibit increased sensitivity to IL-2 [67].

MG patients have a larger number of cells that express IFN- γ or IL-4, indicating that both Th1 and Th2 cells are involved in the pathogenic mechanisms of this disease [68]. The involvement of pro-inflammatory Th1 cells was confirmed by the observation that the number of cells expressing CXCR3 is increased in the thymus and peripheral blood of MG patients. This marker is also increased in lymph node cells in the experimental rat model of MG (EAMG), suggesting that CXCR3 plays a role in the inflammatory process that is associated with the autoimmune response [69].

These data demonstrate that the immune system is activated in MG patients, and the presence of a number of inflammatory signs in these patients raises the possibility that the efficiency of immune regulation mechanisms is compromised.

3.2.3.1. T cells from the MG thymus are out of control. The immunoregulatory defects that are observed in MG patients are due to the impairment of both Treg cells and conventional T (Tconv) cells [70].

CD4+CD25+ Treg cells play an important role in the control of both the autoimmune response and the immune response [71]. Qualitative or quantitative Treg cell defects have been demonstrated in a variety of autoimmune diseases [72]. The number of Treg cells, as defined by the CD25 and Foxp3 markers, was unchanged in the thymuses of MG patients [73,74]. The number of Treg cells in the periphery is also unchanged, whether it is evaluated using the CD25 and Foxp3 markers [75] or the CD25 and CD127 markers [76]. However, in Chinese cohorts, Foxp3 mRNA and protein levels were dramatically down-regulated in peripheral CD4+CD25+ cells [77]. It remains unclear whether this discordance is due to technical differences, such as the source of the anti-Foxp3 antibodies, or to differences in the genetic backgrounds of the tested cohorts.

Although changes in Treg markers in MG patients are still a matter of debate, the function of Treg cells is clearly defective in these patients. We demonstrated that Treg cells from the MG thymus have a severe defect in their suppressive function when co-cultured with autologous CD4+CD25- T cells [73]. This result was also observed using peripheral blood cells [77,78]. More recently, similar results were obtained using the peripheral Treg subset, defined according to the CD25 + CD127- phenotype [76].

The consistent observation of severe immunoregulatory defects in MG patients led us to further analyze the molecular signatures of Treg and Tconv cells using microarray technology. Many genes from the IL-17 family (i.e., IL-17A, IL-17F, IL-21, IL-22, and IL-26) were increased in MG Treg cells compared to control Treg cells [79]. The levels of IFN- γ and TNF- α were also upregulated in MG cells compared to controls for both Treg and Tconv cells. Functional studies suggest that TNF- α plays a role in the chronic inflammation that is observed in the MG thymus [70]. These results emphasize that both Th1 and Th17 cells are involved in the pathologic mechanisms associated with thymic changes in MG patients.

Knowledge concerning the role of the Th17 cell subset in human MG is limited. A recent manuscript reported higher levels of IL-17 in the serum of generalized AChR-MG patients than in control serum

[80]. However, the number of peripheral blood mononuclear cells (PBMCs) expressing ROR γ T was similar in MG patients and healthy subjects [81]. An increase in Th17 cells and Th17-associated cytokines was observed in PBMCs from MG patients with thymomas [82]. Increased production of IL-17 was also described in MG-related thymic hyperplasia [62], confirming our own data [79,70].

Because immunological investigations are performed in MG patients when the disease is already well established, it remains unclear whether the observed Treg cell dysfunction is a primary causal event or a result of perturbations of the immune system that occur during disease development. It is possible that this defect is genetically or epigenetically predetermined; in this scenario, the defective Treg cells would be unable to neutralize an excessive inflammatory reaction. The TEC from MG patients overproduce IL-6 and IL-1 [83,84], and this environment could alter the balance between functional Treg cells and inflammatory cells, particularly Th17 cells. In addition, as suggested above, the Treg cells themselves could be driven to secrete pro-inflammatory IL-17. Thus, the chronic inflammation present in the thymuses of MG patients, which involves a number of cytokines, such as TNF- α and IL-17, clearly contributes to the impairment of T cells and the dysregulation of suppressive activity. A schematic diagram summarizing the mechanisms that lead to thymic inflammation and GC formation in MG patients is shown in Fig. 3.

3.2.4. Effects of thymectomy on the clinical and immunological features of MG

In patients with thymic follicular hyperplasia, thymectomy can change the course of the disease, leading to reduced severity or remission in a significant number of patients [85]. The best results are obtained when the patients have severe thymic hyperplasia and when thymectomy is performed soon after the onset of symptoms [86,87]. This procedure likely eliminates the main site of anti-AChR autoantibody production and often leads to a decreased level of anti-AChR antibodies, which correlates with the number of B cells found in the thymus of the patient [88]. This finding may explain why thymectomy has a better outcome when the patient presents with severe follicular hyperplasia. However, the decreased antibody levels that are observed after thymectomy are not consistent. This finding supports the hypothesis that long lived plasma cells generated in the thymus migrate into the periphery in these patients [48]. The benefits of thymectomy compared to other therapies have been discussed in recent years, and an international randomized clinical trial was established to answer this question [89]. The enrollment of patients was completed in late 2012, but the results will not be available until 2016 because the study includes a 3-year follow-up.

If the improvement that occurs after thymectomy is related to the elimination of thymic B cells, other therapies may produce similar effects. For example, corticosteroids significantly reduce the size and number of GCs in the thymus [52] (Fig. 2). No information is currently available concerning the potential for a rebound effect on thymic B lymphocytes after corticosteroids are discontinued.

Overall, the frequently observed functional and morphological changes of the thymus, the frequent improvement of patients after thymectomy, and the correlation between the degree of follicular hyperplasia and the level of anti-AChR antibodies suggest a causal relationship between thymic pathology and MG.

4. Etiological mechanisms of MG

Regardless of the clinical form, MG is a multifactorial disease. The onset of the disease is not clearly defined and is likely linked to a combination of predisposing factors and environmental factors.

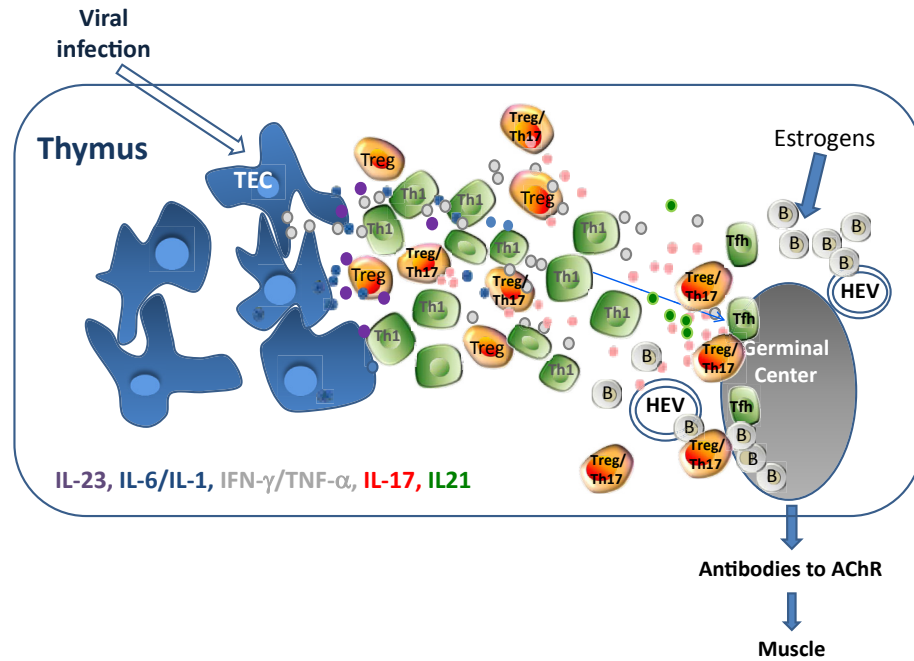


Fig. 3. A model of the pathogenic mechanisms leading to hyperplasia. After a triggering event, such as viral infection, in a genetically favorable background, thymic epithelial cells (TEC) overproduce IL-1, IL-6, and IFN- β and lymphocytes overproduce TNF- α and IFN- γ . These cytokines increase the expression of MHC class II, AChR, and the chemokines CXCL13, CCL21, and SDF-1 (not represented in the model), which contribute to the attraction of B cells from the periphery. Estrogens promote the proliferation of B cells, which could explain why follicles are primarily found in females. This inflammatory milieu modulates the T cell phenotype: Treg cells produce Th17 cytokines, and both Tconv and Treg cells produce IFN- γ , TNF- α , and IL-21. IL-17 and IL-21 favor the development of T follicular helper cells (Tfh) and the generation of germinal centers. In this inflammatory environment, the Treg cells become inefficient, and the Tconv become resistant to suppression. Altogether, this cascade of events leads to thymic hyperplasia and MG.

4.1. Genetic susceptibility to MG

MG families are very rare, and few studies have focused on familial or twin cases. However, a recent study examined the MG literature and determined the concordance rate in monozygotic twins [90]. The study, which compiled 31 pairs of monozygotic twins, showed concordance in 35% of cases (11 couples), while the frequency among heterozygous twins was approximately 4–5%. These results highlight the important role of an individual's genetic background in susceptibility to MG.

The association of human leukocyte antigen (HLA) class I and class II genes with MG is clearly established [91]. The association between HLA-B8 (MHC class I) and -DR3 (MHC class II) and EOMG, which is associated with thymic follicular hyperplasia, was confirmed in several studies (reviewed in Ref. [92]). LOMG is associated with HLA-DR2-B7. Interestingly, the MHC class II genes HLA-DR3 and HLA-DR7 appear to have opposite effects on different subgroups of MG. HLA-DR3 is frequently associated with EOMG and appears to be protective in LOMG patients, while HLA-DR7 has the opposite effects. An association with HLA-DR14-DQ5 was observed in MuSK-MG patients (reviewed in Ref. [93]).

Other susceptibility genes have also been described. Some of these genes are also associated with other autoimmune diseases and represent genes of susceptibility to autoimmunity. These genes include PTPN22, CTLA-4, IL-1 β , IL-10, TNF- α , and IFN- γ [94]. The PTPN22 gene encodes a member of the tyrosine phosphatase subfamily and interferes with signaling in T cells, leading to the inhibition of T cell activation. The CTLA-4 molecule is highly polymorphic and plays an immunoregulatory role by limiting the excessive activation of T cells. A particular TNF- α allele is frequently found in women with EOMG. In addition, this allele is associated with excessive production of TNF- α [95]. The α -AChR promoter is also genetically associated with autoimmune MG (reviewed in Ref. [96]).

A recent study identified a homozygous single nucleotide variant related to the ecto-NADH oxidase 1 (ENOX1) gene in a kindred with parental consanguinity (i.e., 5 of 10 siblings) with LOMG. ENOX proteins catalyze hydroquinone oxidation and protein disulfide-thiol interchange. The ENOX1 variant might predispose individuals to MG by affecting the motor endplate and/or the autoimmune response. However, the exact role of ENOX1 in MG needs to be investigated in larger cohorts of patients [97].

Finally, in a genome-wide association study (GWAS) performed in northern European EOMG patients, in addition to a strong association with the HLA class I (HLA-B*08) region already known, an association with the transcription factor TCF19 was observed in AChR-MG patients [98]. TCF19 is involved in cell proliferation and differentiation and is up-regulated in human pro-B and pre-B cells. This gene is also highly expressed in GC cells [99]. Two other loci were also identified, corresponding to PTPN22 and the TNF- α -induced protein 3 interacting protein 1 (TNIP1). TNIP1 is located in the HLA chromosomal region, and polymorphisms in this gene have been related to chronic inflammatory diseases [100,101]. TNIP1 is a signal transduction protein that inhibits signal transduction by transmembrane and nuclear receptors. This protein is required for the termination of TLR responses [102] and displays inhibitory activities, as its overexpression reduces the TNF- α - or IL-1-induced activation of NF- κ B. Mice with a knockin mutation disrupting the ubiquitin-binding region of TNIP1 developed the hallmarks of autoimmunity, including the spontaneous formation of GCs, isotype switching, and the production of autoreactive antibodies [103]. Altogether, this data suggests that this TNIP1 variant is associated with an exacerbated inflammatory state.

It is worth noting that most of the genes associated with MG are involved in regulating the immune system. We could therefore propose that subjects with specific alleles that are associated with lower immunoregulatory capacity may be more susceptible to autoimmune diseases, particularly to MG. In addition to genetic

analyses, numerous studies are also in progress to determine the impact of epigenetic modifications on selected genes that might play a central role in autoimmune diseases.

4.2. The implication of miRNAs in MG

MicroRNAs (miRNAs) are small non-coding RNAs that mediate post-transcriptional silencing of target genes. In animals, miRNAs typically bind to complementary sites in the 3' untranslated region (UTR) of target genes and regulate target gene expression by translational inhibition, mRNA degradation, or both of these processes [104]. The dysregulation of miRNAs has been described in a variety of autoimmune diseases, including psoriasis, rheumatoid arthritis, systemic lupus erythematosus, and many others [105–108]. However, studies addressing miRNAs in MG are limited.

Recently, 34 miRNAs that are differentially expressed in peripheral blood lymphocytes from MG patients and healthy controls were identified in a Chinese cohort [109]. Interestingly, a decrease in miR-320a levels was observed, and this decrease was correlated with increases in the levels of pro-inflammatory cytokines (i.e., IL-2, IFN- γ , IL-17, and IL-6). miR-320a inhibited the extracellular-regulated kinase (ERK) pathway. The link between changes in the expression of this miRNA and the physiopathological mechanisms of MG requires further investigation. To date, an association between miR-320a and the autoimmune process has not been demonstrated, but this miRNA is frequently down-regulated in cancer cell lines and colon cancer tissues [110] and increased in the sera of patients with heart failure [111].

Significantly decreased levels of let-7 family miRNAs were observed in PBMCs from MG patients, when compared to healthy controls [112]. There was a negative correlation between let-7c and IL-10 mRNA levels. Interestingly, in a recent manuscript, Gandhi et al. demonstrated that the let-7 family of miRNAs differentiated subgroups of Multiple Sclerosis (MS) patients from healthy controls [113]. The let-7 miRNAs were demonstrated to regulate stem cell differentiation and T cell activation and activate TLR7. Let-7 was also demonstrated to regulate Fas expression and sensitivity to Fas-mediated apoptosis [114]. Because Fas is upregulated in T cells from MG patients [66], it is possible that changes in the expression of let-7 could explain the dysregulation of Fas in MG patients. In addition, a link between let-7 downregulation and infection was recently demonstrated. Infection with *Salmonella* downregulated the expression of let-7 miRNAs in several cell types, and lipopolysaccharide (LPS) also fully recapitulated let-7 repression.

These findings highlight promising new research avenues. Changes in miRNA expression that result from external events, such as infections, could significantly alter the regulation of immune cells.

4.3. The role of sex hormones in MG

In patients with EOMG, there is a clear relationship between thymic pathology and gender. Thymic follicular hyperplasia primarily affects female patients, with a male:female ratio of 9:1, during the fecund period of their lives [15], highlighting the potential link between B cell-mediated autoimmunity and sex hormones.

Sex hormones, primarily estrogen but also progesterone and testosterone, affect immune cells quantitatively and qualitatively. Relevant studies have focused on the impact of these hormones on cytokine production by different effector cells and on immunoglobulin production by B lymphocytes [115]. Estrogens typically favor immune processes involving CD4⁺ Th2 cells and B cells, promoting B cell-mediated autoimmune diseases [116]. In the B cell compartment, estrogen is an immune stimulator that affects

maturation, selection, and antibody secretion. It also likely allows autoreactive B cells to escape the normal mechanisms that promote tolerance and accumulate in sufficient numbers to cause autoimmune diseases (reviewed in Ref. [117]). In addition, a link between autoimmunity, the formation of GCs, and estrogen receptors (ERs) has been previously reported. ER α knockout mice exhibit immune complex-type glomerulonephritis, destruction of tubular cells, and severe infiltration of B lymphocytes into the kidney, as well as the spontaneous formation of GCs in the spleen in the absence of antigen challenge [118].

Estrogens also have a strong influence on the development and maintenance of thymic function and thus, on the generation of naive CD4 and CD8 T cells. ER α -deficient mice exhibit both smaller thymus than their wild-type littermates and E2-induced thymus atrophy [119]. However, E2 does not induce lymphocytopenia in the peripheral organs, but instead stimulates extrathymic T cell numbers in the liver and other organs [120]. In addition, physiologic levels of estrogen stimulate the expansion of CD4⁺ CD25⁺ Treg cells, which help maintain tolerance to self-antigens [121]. However, as discussed above (Section 3.2), the Treg cells could be inefficient because of the effects of the inflammatory milieu.

The modulation of the clinical symptoms of MG during pregnancy and menstruation has also been reported, and this phenomenon can disappear after thymectomy [122]. The exacerbation of MG can occur during pregnancy and the postpartum period [123]. It is therefore likely that sex hormones have short-term effects. It is unclear whether sex hormones have direct effects on AChR function. Interestingly, the expression levels of ERs were increased in both the thymus and PBMCs in MG patients. We further demonstrated that ER expression is increased by treatment with pro-inflammatory cytokines [124].

The effects of sex hormones on the development of MG have also been evaluated in the experimental model of MG. Leker et al. observed no significant differences in rats that underwent ovariectomy [125], while Delpy et al. demonstrated that estrogen enhanced susceptibility to EAMG [126] by promoting Th1 immune responses.

Altogether, the available data indicate that estrogen can influence both anti-inflammatory and pro-inflammatory responses. The effects of estrogens are highly dependent on the dose, the timing, and the microenvironment (reviewed in Ref. [117]). Further data are needed to confirm the role of sex hormones in MG. The dysregulation of ER expression in thymocytes and PBMCs from MG patients could contribute to the induction, maintenance, and progression of the autoimmune response via effects on cytokine production and B cell activity.

4.4. Environmental risk factors for MG

Environmental factors, such as drugs (i.e., D-penicillamine and IFN-I), pollutants, and pathogens, are also proposed to increase the risk of developing an autoimmune disease [96]. The hypothesis that an infectious agent is involved in the pathogenesis of MG has also been proposed, as the thymus is sensitive to infections [127]. An association between viral infection and thymic pathology has been suggested. The presence of B cells with signs of Epstein Barr virus (EBV) infection was found in the hyperplastic MG thymus but not in control patients [128]. The role of these cells in the development of MG remains unclear, and further studies are needed to determine whether EBV plays a role in the triggering events that lead to MG or in the events that promote the chronic nature of the disease. Several reports indicate that other viruses, such as cytomegalovirus, human foamy virus, and Nile virus, are associated with MG [96,129]. Because MG symptoms likely occur long after a possible

triggering infection, it is difficult to link MG with a particular infection.

An antiviral signature can be observed in the MG thymus. Numerous IFN-I-induced genes are overexpressed in the thymuses of MG patients [58,130]. The MG thymus also exhibits overexpression of IFN- β and TLR4 [131], as well as double-stranded (ds) RNA signaling molecules, including TLR3, protein kinase R, IFN-regulatory Factor (IRF)5 and IRF7. Recently, Poly(I:C), a synthetic analog of dsRNA, was demonstrated to specifically induce thymic overexpression of α -AChR. Overexpression of other AChR subunits or tissue-specific antigens was not observed. This overexpression is mediated by TLR3, protein kinase R and the release of IFN- β . When Poly(I:C) is injected into mice, it causes an increase in the number of B lymphocytes in the thymus and the appearance of anti-AChR antibodies in the periphery. At the same time, the expression of AChR in the diaphragm muscle and clinical symptoms of muscle weakness are reduced. Because anti-AChR antibodies are both highly specific for MG and pathogenic, the activation of dsRNA signaling could contribute to the etiology of MG [132]. dsRNA is the genetic material of certain viruses, and it is also produced during the replication cycle of many viruses. Thus, these data strongly suggest that MG develops after viral infection. This observation could also be related to the presence of EBV-positive cells in the MG thymus [133]. EBV encodes small RNAs (EBER) that trigger TLR3 signaling and induce IFN-I and pro-inflammatory cytokine expression, similarly to Poly(I:C) [134]. In addition, a recent study reported higher levels of antibodies against the type 1 nuclear antigen of EBV in the serum of EOMG patients than in healthy controls [135]. Changes in the level of expression of TLRs have also been detected in PBMCs, suggesting that TLRs contribute to MG pathogenesis. The consequences of these changes and their role in MG remain unclear. However, a positive correlation between TLR9 mRNA levels and the clinical severity of MG has been observed [136].

Several findings suggest that IFN-I is implicated in MG: 1) clinical reports demonstrate the development of MG after IFN- α - or - β -based therapy [137–140]; 2) antibodies against IFN-I are found in some MG patients [41,141]; and 3) as indicated above, thymic transcriptome analysis from different MG patient subgroups revealed a significant increase in the expression of IFN-I-induced genes [58]. It was also recently demonstrated that IFN-I, particularly IFN- β , could play a central role in the thymic follicular hyperplasia that occurs in MG patients. IFN- β induces the overexpression of α -AChR in TECs and of the chemokines CXCL13 and CCL21 in TECs and endothelial lymphatic cells, respectively. IFN- β also increases the expression of B cell activating factor (BAFF), which favors autoreactive B cells [142].

An association between the development of thymoma and viral infection has also been suggested recently, and one paper in this issue presents data relevant to this hypothesis. Patients with thymoma possess high levels of anti-IFN-I neutralizing antibodies, and these neutralizing antibodies are also detected in response to viral infections [41]. It was recently observed that the thymuses of MG patients with thymomas exhibit high levels of IFN-I, particularly IFN- α 2, IFN- α 8, IFN- ω , and IFN- β , and dysregulated expression of dsRNA signaling molecules [42]. Altogether, these observations suggest that the development of thymomas in MG patients could be related to a viral infection.

5. Conclusion

MG has been actively studied since the 1970s, especially following the discovery of anti-AChR autoantibodies. However, recent investigations have improved our understanding of MG and highlighted new questions related to the development of MG. New

antigenic targets have been described, and an improved classification system for the different MG subtypes and their distinct physiopathological mechanisms has been delineated. Recent microarray analyses have revealed a number of mechanisms that are shared between MG and other autoimmune and inflammatory diseases. In addition, these studies demonstrated that thymuses from EOMG patients exhibit a pro-inflammatory environment that could explain the observed dysregulated T cells and chronic immune activation. The cytokines IFN- γ , TNF- α and IL-17 play a central role in these mechanisms. New developments have been reported regarding the role of miRNAs in the modulation of immune function, and these findings warrant future investigation. These findings are particularly interesting in the context of the etiological mechanisms of MG, as the activation of dsRNA signaling pathways has been demonstrated to induce MG in mice.

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