Review

Etiology of myasthenia gravis: Innate immunity signature in pathological thymus

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ABSTRACT

Myasthenia gravis (MG) is an autoimmune disease affecting the neuromuscular junction (NMJ), whose clinical hallmark is muscle weakness and early fatigability. The main target of autoimmunity in MG is the acetylcholine receptor (AChR) located in the NMJ. It is now widely accepted that the thymus is probably the prime site of autoimmunity development and maintenance in AChR-positive MG patients; however, the exact mechanisms triggering and perpetuating the intra-thymic autoimmune response to AChR are still unknown. As with many autoimmune diseases, MG has a multifactorial etiology, resulting from complex interactions between genetic and environmental factors, as fully described in this review. Among environmental factors, viral infections could play a central role in autoimmunity, mainly through the induction of dysregulated Toll-like receptor (TLR)-mediated innate immune responses, which can lead to inflammation and adaptive autoimmune response. Growing evidence of chronic inflammation, TLR activation, and persistent viral infections in the thymus of MG patients, strongly supports the hypothesis that, in the context of a genetic susceptible background, the intrathymic innate immune responses to pathogen infections might contribute to MG etiology.

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Abbreviations: AChR, acetylcholine receptor; EAMG, experimental autoimmune MG; EBV, Epstein–Barr virus; ENOX1, ecto-NADH oxidase 1 gene; EOMG, early-onset MG; GC, germinal center; GWAS, genome-wide association studies; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; LRP4, lipoprotein receptor-related protein 4; LOMG, late-onset MG; MG, myasthenia gravis; MuSK, muscle specific kinase; NMJ, neuromuscular junction; PTPN22, protein tyrosine phosphatase nonreceptor-22; PV, poliovirus; TLR, Toll-like receptor; TNF- α , tumor necrosis factor- α ; TNIP1, TNF- α -induced protein 3 (TNFAIP3)-interacting protein 1.

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1. Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction (NMJ) in which muscle weakness and abnormal fatigability on exertion are the prominent clinical features; symptoms are due to neuromuscular transmission impairment caused by autoantibodies targeting postsynaptic membrane proteins of the striated skeletal muscles. Over 80% of patients have antibodies against acetylcholine receptor (AChR) [1] or clustered AChRs [2,3] and variable proportions (37.5–70.0%) of AChR-negative patients have antibodies to the muscle kinase receptor (MuSK) [4–10]. Recently, autoantibodies to the lipoprotein receptor-related protein 4 (LRP4) have been detected in variable proportions of AChR- and MuSK-negative (seronegative) MG patients [11–13] (Table 1).

MG patients can be distinguished in purely ocular and generalized forms and in early-onset (EOMG; under 40 years old) or late-onset (LOMG; over 40 years old) forms. At onset, extraocular muscles are often the first ones to be affected and may remain the only muscles involved (pure ocular MG); however, in many cases ocular disease progresses and involves skeletal or bulbar muscles, giving rise to the generalized form of MG [1]. Distribution of age at MG onset shows a female-dominant peak in EOMG and a male-dominant peak in LOMG [14]. In the recent years, the incidence of MG in the elderly (over 50 years old) population is increasing, likely due to aging, improved recognition and diagnosis of the disease or to unknown environmental factors [15–17].

In AChR-MG, correlation between disease severity and anti-AChR antibody titers is poor, hence the antibody prognostic value is poor [18]. MuSK-MG patients present often with more severe symptoms than AChR-MG patients. They have prominent bulbar and facial weakness associated with marked muscle atrophy and are less responsive to treatments [6]. Seronegative MG patients are instead clinically indistinguishable from AChR-MG patients [19].

Growing evidence supports the involvement of the thymus in the pathogenesis of MG. The role of this organ as a prime site of autosensitization and autoimmunity is widely accepted in AChR-MG patients. In these patients, the thymus exhibits morphological and functional abnormalities, usually characterized by a thymoma or by hyperplasia [20,21]. In MuSK-MG patients, the thymic contribution to the disease remains unclear (see Section 3 for an extensive review of the thymic MG pathogenesis).

Like other autoimmune diseases, MG is multifactorial and its manifestation results from the combination and interaction of multiple genetic and environmental risk factors. AChR-MG occurring in EOMG patients and presenting with thymic hyperplasia is the most common, and also the best characterized, subtype of MG. Although anti-AChR autoantibodies have been identified almost forty years ago [22], the exact immunological mechanisms underlying the autoimmune response are still unknown. Current treatments are quite effective but complete stable remission is observed only in a proportion of patients; these observations highlight the need of better understanding the specific factors and events initiating or perpetuating the disease [23]. A more comprehensive understanding of the genetic and environmental factors associated with MG and demonstration of the pathologically relevant gene-environment interactions are crucial for improving our knowledge in MG pathogenesis.

In this manuscript, we review early and more recent data on genetic and environmental risk factors associated with MG, giving

particular emphasis on the emerging role of viral infections and innate immunity in the intrathymic pathogenesis of the disease.

2. Genetic and environmental factors related to MG etiology

MG is a multifactorial disease and appears likely to be linked to a combination of predisposing factors and triggering event(s). In thymoma-associated MG, the development of thymic epithelial tumors is a clear pathogenetic event, even if thymoma is not always associated with MG. In the other forms of autoimmune MG, the event leading to the disease is not clearly defined.

2.1. Genetic factors

Autoimmune MG does not show any Mendelian hereditary. However, as for many autoimmune diseases, a genetic risk accounts for some responsibility in the disease development, as attested by the 40% concordance rate in monozygotic twins [24].

2.1.1. The HLA genomic region

The association of human leukocyte antigen (HLA) class I and class II genes with MG is clearly established [25]. These genes comprise several hundreds of alleles on the chromosome 6p21 region, encoding membrane-bound molecules of the immunoglobulin superfamily that present antigenic epitopes to lymphoid cells. In Caucasian MG patients, a strong genetic association is observed between the HLA A1–B8–DR3 haplotype and EOMG patients [26]. The HLA-DR3 and B8 alleles are part of the most conserved HLA haplotype in European populations, the “so-called” 8.1 haplotype that associates the HLA-B8 allele with DR3, along with alleles of many other HLA loci, such as HLA-A1. This haplotype has been associated with several autoimmune conditions, including rheumatoid arthritis [27] and systemic lupus erythematosus [28], which often co-occur in MG patients [29]. The 8.1 haplotype was found to have a strong and complex genetic effect on phenotype in EOMG associated with thymic hyperplasia, including high serum titers of AChR autoantibodies [25]. However, because of strong linkage disequilibrium extending across this haplotype, the causative alleles are not known yet. Some studies pointed to a major contribution of HLA-DQ alleles to MG, as DQB1*0502 which has been associated with MG Italian population [30]. A recent study demonstrated novel associations between HLA-DQ loci and EOMG in southeast Texas patients, not previously reported in European MG patients, suggesting that region-specific environmental factors may interact with HLA genes to predispose toward MG [31]. The role of this specific HLA haplotype in MG is not clear. Nevertheless, peripheral blood mononuclear cells from HLA-B8, DR3-positive and DR3-negative individuals differ in their ability to produce interleukin (IL)-2, IL-5, IL-12 and interferon type II (IFN-II) upon stimulation [32].

Other genetic associations have been recently pointed out for different MG forms. A study has demonstrated a genetic association between the HLA DRB1*15:01 haplotype and LOMG patients [33]. In MuSK-MG, the disease has been associated with the HLA-DR14–DQ5 haplotype in a Dutch cohort [34] and with the HLA-DR14–DQ5 and HLA-DR16–DQ5 haplotypes in an Italian population [35], suggesting a major role for the DQ5 allele in this MG subtype.

In MG patients with thymoma, studies associating MG with HLA I and II alleles did not give reproducible results [36,37]. A recent

Table 1

Autoantibody profile in MG patients: summary of the key features.

MG form	AChR	MuSK	LRP4	Clustered AChR
% of antibody positive patients	80–85%	5–8%	~2–3%	~5%
Antibody pathogenicity	Tested in animal model and in vitro	Tested in animal model and in vitro	Tested in vitro	Tested in vitro
Isotypes	IgG1, IgG3	IgG4	IgG1	–
Role of complement	Yes	No	Yes	Likely
Correlation with disease severity	No	Yes	Unknown	Unknown
Thymic pathology	Hyperplasia in most EOMG; Thymoma or thymic atrophy in most LOMG	Involved thymus resembling those of age-matched controls	Unknown	Likely similar to AChR-MG

investigation of the association of the HLA-A locus in French Caucasian patients with thymoma revealed a protective effect of HLA-A02 in patients with a B2 type thymoma (World Health Organization classification) and the increased frequency of HLA-A25 in all the MG patients with thymoma analyzed [37]. In northern Chinese population, HLA-DQA1*0401 and HLA-DQB1*0604 were recently associated with thymoma-associated MG and it was suggested that different genetic mechanisms may characterize MG patients with and without thymoma [38].

Interestingly, polymorphisms on genes, different from HLA class I and II, located in the 6p21 chromosomal region, are also associated with MG susceptibility. This is the case for the tumor necrosis factor (TNF- α) locus: peripheral blood mononuclear cells (PBMC) from patients with specific TNF- α polymorphisms showed higher secretion of TNF- α when activated in vitro [39,40]. Interestingly, TNF- α was shown to be also implicated in the development of EAMG [41].

2.1.2. Non HLA-related genes associated with MG

Polymorphisms on genes in chromosomal regions different from 6p21 have been associated with MG, although they are also often associated with other autoimmune diseases: these are cytotoxic lymphocyte-associated protein-4 (CTLA4) [42], IFN- γ , IL-10, IL-12 [43] and protein tyrosine phosphatase nonreceptor-22 (PTPN22) [44,45]. The rs2476601 (R620W) functional variant in the PTPN22 gene, which encodes a tyrosine phosphatase primarily expressed in lymphoid tissues, was associated with several autoimmune conditions [46,47] and showed ability to interfere with autoreactive B-cell removal and increase T- and dendritic-cell responsiveness [47–49]. In MG, no reproducible associations were obtained in French and Italian patients: the PTPN22 rs2476601 variant was associated with MG in French population [50] but not in Italian population, in which the variant was not associated with either MG itself or any MG characteristic [51]. However, another PTPN22 polymorphism, the rs2488457 variant of the promoter region, was associated with low autoantibody titers and mild MG in the Italian cohort, suggesting a role of this variant in MG [51]. Recently, the PTPN22 gene was also reported as a risk factor for MG associated with thymoma: the PTPN22 gain-of-function 1858 T variant correlated with low IL-2 expression in thymomas, thus implying weaker T-cell receptor signaling. This result suggested that the 1858 T variant may predispose to MG in a subgroup of thymoma patients possibly by impeding central tolerance induction [52].

Among non-HLA loci, of particular interest is the locus coding for α -AChR that is the main target of autoantibodies in MG patients. Giraud et al. identified a functional bi-allelic variant in the promoter of the α -AChR which is associated with EOMG patients. This variant prevents binding of interferon regulatory factor 8 (IRF8) and decreases α -AChR promoter activity in thymic epithelial cells in vitro. In a French cohort of MG patients, this variant appears to be more frequent in MG patients with an odds ratio of 2.35, but only in patients with an early onset of disease (under 21 years old) [53]. This decrease

could alter central tolerance and lead to the escape of AChR-specific autoreactive T cells from the thymus.

2.1.3. Genome-wide association studies on MG patients

Genome-wide association studies (GWAS) aimed at analyzing common genetic variations across the entire human genome in an unbiased manner in order to identify genetic risk factors associated with specific diseases. In a review on GWAS carried out on autoimmune disorders, Lettre et al. underline the discovery of polymorphisms on genes related to both adaptive immunity and innate immunity, and in particular on IL and IL receptor genes [54]. A GWAS has been very recently carried out on 649 North European EOMG patients. As expected the strongest association was found within HLA loci and, in particular, in a HLA class I region (rs7750641) that encodes TCF19. TCF19 is one of the transcription factors involved in cell proliferation or differentiation being up-regulated in human pro-B and pre-B cells [55]. It is also highly expressed in germinal center (GC) cells. This locus has also been associated with type I diabetes [56]. Moreover, from this GWAS two loci were also extracted: rs2476601 corresponding to PTPN22, already described above (Section 2.1.2), and rs4958881 for TNF- α -induced protein 3 (TNFAIP3)-interacting protein 1 (TNIP1, also known as ABIN-1, Naf1 and VAN). A growing number of references has implicated TNIP1 in chronic inflammatory diseases, such as systemic lupus erythematosus and psoriatic arthritis, through GWAS and expression studies [57,58]. TNIP1 is a signaling transduction protein that inhibits both transduction by transmembrane receptors, such as TNF- α receptor, epidermal growth factor receptor and Toll-like receptors (TLRs), and nuclear receptors, such as peroxisome proliferator activated receptors (PPARs) and retinoic acid receptors (RARs). These receptors play key roles in regulating inflammation and inflammatory diseases [59]. Mice with a knock-in mutation disrupting the ubiquitin-binding of TNIP1 developed the hallmarks of autoimmunity, including spontaneous formation of GC, isotype switching, and production of autoreactive antibodies. Autoimmunity was suppressed by crossing to MyD88^{-/-} mice, demonstrating that TLR-MyD88 signaling pathways are needed for the phenotype to develop [60]. However, the polymorphism observed in the GWAS on TNIP1 in MG patients is not located in the ubiquitin-binding or protein interaction regions. In other inflammatory diseases where TNIP1 has been involved, the polymorphisms observed were spotted on different sites. The exact role of TNIP1 remains to be elucidated both in normal physiology and in diseases.

Another large genomic study of SNPs on candidate genes on about a thousand of EOMG patients is currently in progress and might also shed light on other genes associated with a higher MG susceptibility risk.

Homozygosity mapping is an efficient gene mapping method applicable to rare recessive disorders in inbred populations. A genome-wide homozygosity study has been performed in kindred with parental consanguinity (5 of 10 sibs) with LOMG [61]. A homozygous single nucleotide variant was found in the 3'-untranslated region of the ecto-NADH oxidase 1 gene (ENOX1) in 4 affected sibs and 1 unaffected

sibling, while the ENOX1 sequence variant was not found in 764 controls. ENOX proteins are growth-related cell surface enzymes that catalyze hydroquinone oxidation and protein disulfide–thiol interchange. In principle, the ENOX1 mutation might predispose to MG by affecting the motor endplate and/or the autoimmune response. However, it is not clear how a genetically mediated reduction in the amount of ENOX1 efficiently causes LOMG development and the exact role of ENOX1 remains to be investigated in larger cohorts of MG patients [61].

Growing numbers of novel susceptibility loci have been identified in autoimmune diseases through the development of high-throughput genotyping techniques. Now the path to understand the link between changes in genome sequences and disease predisposition, progression and/or chronicity might still be long. Beyond pure 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.

2.2. Gender influence

As for many autoimmune diseases, there is a gender bias in MG patients [62,63]. MG is more frequently observed in women with a 2–4:1 female bias according to studies. In EOMG with thymic hyperplasia, the bias is even stronger with a 9:1 ratio [64]. What can predispose women to autoimmunity and in particular to MG? Hormonal or genetic effects have been debated. Steroid hormones, namely estrogen, progesterone and testosterone, may influence different cells of the immune system by modulating cell proliferation, cytokine production and antibody production [65]. An enhanced susceptibility to EAMG is observed in C57BL/6 mice treated with 17 β -estradiol before antigen (T-AChR) priming [66]. In contrast, knock-out mice for the estrogen receptor- α are equally susceptible to EAMG as wild-type C57BL/6 mice suggesting that increased susceptibility to EAMG is more likely related to increased levels of estrogen than a decrease. However, EAMG model does not reflect properly the human disease as it does not resume thymic changes of MG patients. Hormones could influence thymopoiesis and affect central tolerance. Estrogen receptors are expressed on thymocytes and thymic stromal cells and estrogen decreases thymus size [67,68]. We found an increased expression of estrogen receptor- α on thymocytes from MG patients that, consequently, could be more sensitive to the effects of estrogen. This up-regulation could be due to the inflammatory environment characteristic of the thymus of MG patients [68].

A role of sex chromosomes in autoimmunity has also been proposed including several possible mechanisms such as fetal microchimerism, X-chromosome inactivation patterns, and X-chromosome duplication, but so far nothing has been related to MG [69].

2.3. Drug-induced MG

D-penicillamine is a metabolite of penicillin, although it has no anti-biotic properties. It is used, as a chelating agent to treat Wilson's disease, but also as an immunosuppressor to treat rheumatoid arthritis. However, the use of D-penicillamine has been associated with various immune-mediated complications, including MG. MG is reported to occur in 1% to 7% of all patients on D-penicillamine and discontinuation of the treatment leads usually to complete resolution of MG [70]. The symptoms in D-penicillamine-induced MG are usually mild but clearly associated with the presence of anti-AChR antibodies. However, a recent study reports the development of an anti-AChR and anti-MuSK MG after D-penicillamine treatment in a patient with scleroderma [71]. The mechanisms involved in D-penicillamine effects are unclear. A specific genetic susceptibility in the HLA class II region (DR1 and/or DR7) has been suggested in patients with a D-penicillamine-induced MG form [26].

Numerous case reports in the medical literature illustrate the fact that IFN- α or IFN- β therapies can trigger the development of certain

autoimmune diseases, including MG [72]. The implication of IFN- α / β in MG has long been suggested in various ways but never clearly defined. We recently demonstrated that IFN- β could play a central role in MG development: IFN- β , which is overexpressed in the thymus of MG patients, can mediate the effects of dsRNA activation and trigger a specific overexpression of α -AChR subunit [73] (see detail in Section 4.2).

2.4. Environmental risk factors

Numerous factors have been put forward in the last decade to explain the higher risk of developing an autoimmune disease in industrialized countries: these include pollution, better hygienic conditions, and microbiota changes [74]. None of them has been clearly addressed in the case of MG. Nevertheless, a recent study has revealed that plasma levels of vitamin D are significantly lower in patients with MG compared with healthy controls [75] suggesting that vitamin D deficiency could affect MG risk and progression, as in multiple sclerosis [76] and other autoimmune diseases [77].

Many MG patients, when asked if they can relate the onset of disease to “something”, can also quote different environmental factors, such as stress, loss of a close relative, fatigue, and infections.

Pathogens are major environmental factors for driving/perpetuating autoimmunity [74,78]. Viral infections have been associated with the development of several autoimmune diseases, mainly by sero-epidemiologic and immunologic studies [79]. For example, type 1 diabetes has been associated with coxsackievirus and cytomegalovirus, multiple sclerosis with Epstein–Barr virus (EBV) and measles virus, and rheumatoid arthritis and systemic lupus erythematosus with EBV [78]. However, since the onset of an autoimmune disease can occur well after a possible triggering infection, when the pathogen might have already been cleared or the antiviral immune responses might have subsided (“hit-and-run” hypothesis) [78], the link of infections with autoimmune diseases has been elusive. Nevertheless, accumulating evidence tends to support the idea of a contribution of viruses in promoting, exacerbating or maintaining autoimmune conditions [80].

In the following sections, after a general overview on the thymic pathology in MG patients, we will discuss the role of innate immunity in autoimmunity and the emerging contribution of viral infections and innate immune system to the intra-thymic pathogenesis of AChR-MG.

3. Thymus pathology: a dominant feature of AChR-MG

Pathological alterations of the thymus are found in approximately 80% of AChR-MG patients with generalized disease (Table 1). The most frequent change is thymic hyperplasia, observed in 50–60% of these patients, thymoma represents approximately 15% of patients and 10–20% of AChR MG cases have an atrophic or involuted thymus mainly consisting of adipose tissue with residual areas of thymic parenchyma [20]. Hyperplasia is the most common alteration in EOMG, whereas thymoma and involuted thymus are predominant in LOMG. In MuSK-MG patients no thymic histological alterations are usually found; thymectomized seronegative MG patients reveal thymic hyperplastic changes very similar to those found in AChR-MG patients (Table 1) [81,82].

3.1. Thymoma

Thymoma in the vast majority of cases is a slow-growing, capsulated epithelial tumor of the thymic gland, commonly presenting in middle or old age; infrequently thymoma can develop at young age and behave as a locally invasive tumor. Thymoma is frequently associated with the presence of autoimmune diseases, with MG occurring in about 30% of all patients with thymomas [83]. Thymic epithelial neoplasms mostly consist of transformed epithelial cells, surrounded

by numerous maturing polyclonal T cells (thymocytes). The immature T cells are doubly positive for CD4 and CD8, a phenotype that characterizes T cells in the cortex of the normal thymus. Type B2 thymoma (World Health Organization classification) [84] is most often associated with MG, followed by types AB and B1 [85].

The fact that MG patients with thymoma have always anti-AChR antibodies strongly suggests that the neoplastic transformation of thymus plays an important role in the initiation of the autoimmune response to AChR. Features of thymoma that may explain T-cell selection impairment and anti-AChR autoimmunity are: a) the lack of a functional tolerogenic autoimmune regulator (AIRE)-positive thymic medulla (in types A, AB, most B2 and B3 tumors) and the occurrence of thymic cortex-like areas (in type B1 thymomas) [86]; b) the presence of a thymic epithelium defective in the expression of HLA class II molecules [87]; c) the lack or reduction of tolerogenic AChR-positive thymic myoid cells; and d) failure to generate FOXP3-positive regulatory T cells [83,88].

3.2. Thymic hyperplasia

The hyperplastic thymus is characterized by the presence of B lymphocyte infiltrates invading the thymic medulla or present in perivascular spaces that are fused with the thymic medulla. B-cell infiltrates can be present in the form of ectopic B-cell follicles with inclusion of GCs (follicular hyperplasia) or can be distributed throughout the medullary parenchyma (diffuse hyperplasia or thymitis) [20,89]. An overall increased expression of immunoglobulin genes, independent of antigenic specificity has been observed in MG thymuses demonstrating the large diversity of B cells in MG thymuses [90,91]. This observation is supported by the observation that thymic GCs contain B-cells undergoing somatic hypermutation and antigen-driven selection producing a heterogeneous population of B cells [92]. The thymus of MG patients has been shown to include all the components of the anti-AChR response including B cells producing anti-AChR antibodies [93]. In hyperplastic MG thymus, GCs are usually surrounded by or contain muscle-like myoid cells that express complete adult and fetal-type AChR [94] or other muscle proteins [95]. Myoid cells, along with thymic epithelial cells, that express isolated AChR subunits [96], are suspected to play a direct role in intra-thymic auto-sensitization to AChR, by driving abnormal antigen presentation or cross-presentation through professional antigen presenting cells. Complement activation has been demonstrated on thymic epithelium and myoid cells in close proximity to GCs in hyperplastic thymus suggesting that autoantibody attack and complement-mediated damage to myoid cells could be responsible to favor autoantigen (i.e. AChR) availability to antigen presenting cells, and predisposing to GC formation and antibody diversification; all these events could promote the generation of self-sustained AChR-specific autoimmune response [97]. Autoreactive T-cells [98] and plasma cells producing anti-AChR antibodies [99] have been isolated from hyperplastic MG thymuses, suggesting that in AChR-MG patients the autoimmune effector arms are actively operating in the thymic gland. The role of thymus in the generation of an autoimmune response to AChR is also supported by the observation that anti-AChR antibody titers in MG patients correlated with the degree of thymic hyperplasia, as hyperplastic patients have higher autoantibody titers than those with involuted thymus or thymoma [100]. Moreover, anti-AChR antibody production by thymic lymphocytes from MG patients correlated with *in vivo* AChR antibody titers and histological thymic abnormalities, with lymphocytes from hyperplastic thymuses being able to produce larger quantities of autoantibodies than those from involuted and thymomatous thymuses [101].

The pathogenic role of the thymus in MG pathogenesis is even reinforced by experiments on severe combined immunodeficiency (SCID) mice grafted with thymic tissue fragments from MG patients. In these mice, human anti-AChR antibodies were detected in serum

1 to 2 weeks after transplantation, proving that the thymus contains all cellular components required for autoantibody production [102].

The evidence that a high proportion of AChR-MG patients undergo true remission after thymectomy [103,104], the fact that AChR antibody titers frequently fall after thymectomy and that the magnitude of this fall correlates with the number of GC B cells in the removed thymus [105], and the observation that the severity of follicular hyperplasia and density of lymphoid infiltrates correlates with improvement after thymectomy [106], again support the pathogenic role of the thymus in AChR-MG [21].

Diffuse hyperplasia (or thymitis) shows histological features similar to those of follicular hyperplasia, with the presence of abundant and diffuse B-cell infiltrates in the thymic parenchyma, but lacks GCs. The involuted thymus that occurs most often in LOMG patients, is very similar to that of age-matched controls in terms of amount of adipose tissue and epithelial space, but the residual islands of medullary parenchyma can present diffuse B-cell infiltrates that in some cases form GCs [89]; this suggests that involuted thymuses are characterized by significant immunological activity.

What drives infiltration of peripheral B-cells and formation of lymphoid aggregates in MG thymus is not fully clear. We demonstrated the overexpression of chemokines in the thymus of MG patients that probably support the recruitment of peripheral cells into the thymus: CXCL13, CCL21, CCL19 and CXCL10 [90,107–109]. We also showed that the hyperplastic MG thymus is the site of active neoangiogenic processes, including the abnormal development of lymphatic vessels and high endothelial venules (HEVs), specialized post-capillary swellings bearing surface chemokines that regulate homing of lymphocytes and dendritic cells in secondary lymphoid organs. Specifically, we demonstrated that CCL21, a chemoattractant for T- and B-cells, is overexpressed in afferent lymphatic endothelial vessels [108]. SDF-1 (also called CXCL12), a chemokine able to recruit monocytes/macrophages, dendritic cells and B-cells is selectively expressed by HEVs of hyperplastic MG thymus [110]. These data suggest a functional role of these chemokines in sustaining the immune cell increase associated with thymic hyperplasia in MG patients.

3.3. The inflammatory status of MG thymus: the IFN imprinting

In MG pathogenesis, as the autoimmune sensitization against AChR is observed in the thymus, the role of inflammatory cytokines is crucial. Thymomatous and some LOMG patients present high-titers of neutralizing autoantibodies against IFN-I and IL-12, suggesting an increased expression of these cytokines. The production of these neutralizing antibodies seems directly linked to the tumors as their titers usually increase strikingly when thymomas recur or metastasize; moreover, cells cultured from thymomas spontaneously produce antibodies to IFN- α and/or IL-12 [111,112]. In EOMG patients, a thymic transcriptome analysis has also underlined the inflammatory state of MG thymus and, in particular, the overexpression of numerous proinflammatory cytokines, chemokines and IFN induced-genes that could participate at different levels to host defense and/or disease in response to viral infection [90]. The potential role of IFN subtypes in EOMG is described below.

IFN-II or IFN- γ is the unique ligand for a receptor consisting of IFN- γ R1 and IFN- γ R2 subunits [113]. IFN-II has been implicated in various autoimmune diseases because of its pro-inflammatory properties, in particular its ability to induce HLA class I and II gene expression and to promote Th1 differentiation. The role of IFN-II has been well studied in MG where it can trigger functional disruption of the neuromuscular junction [114]. Attempts to induce EAMG in IFN-II or IFN-II-receptor knock-out mice demonstrate that IFN-II is involved in the generation of a pathogenic anti-AChR humoral immune response and for conferring susceptibility to clinical EAMG to mice [115,116].

In the thymus, an up-regulation of IFN-II-induced genes is observed and the analysis of the effects of IFN-II on AChR subunit expression in thymic epithelial cell and myoid cell cultures has demonstrated that IFN-II is a strong inducer of AChR subunits and, in particular, of AChR- α [117]. In IFN-II knock-out mice, the absence of IFN-II significantly reduces the expression of AChR- α mRNA in the thymus, while it does not have any effect on AChR- α expression in the muscle [117]. An increased expression of IFN-II that was shown to affect the generation of regulatory T cells could also play a role in MG pathogenesis [118].

IFN-I subtypes are secreted by various cells as an anti-viral defense mechanism. Abnormal production of type I interferon has been associated with multiple autoimmune diseases. The role of IFN-I has not been studied in EAMG models and not so much in human MG pathogenesis. This is probably due to the complexity of the IFN-I family. IFN-I consists of 12 different IFN- α subtypes in addition to one IFN- β and IFN- ω , - ϵ , - τ , - δ and - κ . All IFN-I subtypes have antiviral properties and bind to a common receptor [113].

The implication of IFN-I in MG has long been suggested, as: a) the development of MG was observed after IFN- α - or - β -based therapies [72]; b) antibodies against IFN- α were found in some MG patients, mainly those with thymoma [111,119]; and c) thymic transcriptome analysis of different MG patient subgroups revealed a significantly increased expression of IFN-I-induced genes [64,117]. We recently demonstrated a specific overexpression of IFN- β in the thymus of MG patients [73]. IFN-I could directly affect thymic epithelial cell behavior but also thymocytes differentiation/activation. A study on PBMCs has demonstrated that a chronic elevation of IFN- α could modify the effector/regulatory T cells ratio towards effector T cells and autoimmunity [120]. IFN-I could also alter central tolerance towards α -AChR by modulating its expression in thymic epithelial cells [73].

IFN-III subtypes have been described more recently and comprise IFN- λ 1, - λ 2 and - λ 3, also referred to as IL-29, IL-28A and IL-28B, respectively [113]. These cytokines are induced in response to viral infections and appear to use the same pathways as those of IFN- α / β to develop an anti-viral response [121]. They also seem to act primarily on epithelial cells [122]. In our thymic transcriptome study, these cytokines were not spotted on the arrays but we observed an overrepresentation of the 19q13 chromosome region, the loci of the three IFN-III subtypes [90]. Analyzing by real-time PCR their thymic expression, we observed a significant overexpression of IFN- λ 1/2 but not of IFN- λ 3 in the thymus of MG patients compared to non-MG patients (unpublished data). Their role in MG needs them to be investigated.

The inflammatory environment characteristic of MG thymus, with IFN overexpression, could act at different thymic levels modifying AChR expression, altering self-tolerance, affecting the generation of regulatory T cells, and consequently triggers an autoimmune response towards AChR.

4. Link between innate immunity and autoimmunity in MG

Although the adaptive immune system has long been a focus of attention in the study of autoimmunity, innate immune response to infections is increasingly emerging as being central to the pathogenesis of autoimmune diseases. In MG, striking evidence of chronic inflammation and emerging data on persistent viral infections in the thymus of MG patients strongly support the hypothesis that the innate immune system may trigger or favor the autoimmune condition.

4.1. Overview of the role of innate immunity in autoimmunity

Innate immune system is the first line of defense against infections. It provides a rapid host defense that precedes the adaptive immunity. The main features of the innate immune response include the

following: a) it is nonspecific, being triggered by molecules shared by groups of related pathogens; b) it is immediate and lacks of memory; and c) it is mediated by germline-encoded receptors, the “so-called” pattern recognition receptors (PRRs), that are expressed by the effector cells of innate immunity, mainly natural killer cells, macrophages and dendritic cells, but also by lymphoid cells and non-lymphoid cells. The adaptive immune response is, instead, characterized by relatively slow response kinetics, memory and specificity [123]. Innate and adaptive immune systems have long been studied and considered as two separate entities. However, it has now become clear that the two systems are closely linked and form a single, highly integrated set of defenses against pathogens, with the innate immune response being able to elicit the adaptive immune response [124]. A major role in innate immunity is played by TLRs, a type of PRRs able to recognize conserved microbial molecules and to initiate MyD88-dependent or -independent signaling pathways that lead to inflammatory and antimicrobial innate immune responses. Recognition of microbial products by TLR expressed on dendritic cells triggers activation and functional maturation of these cells into antigen-presenting cells leading to initiation of antigen-specific adaptive immune responses [124,125]. In humans, ten functional members of TLR family have been identified, each of them able to recognize specific microbial derived molecular structures (Table 2) [126]. Although the function of TLR-mediated innate responses is to confer self-protection, several studies, both in experimental settings and in a number of human autoimmune diseases, have indicated that the loss of regulation of TLR signaling, as well as the recognition of self molecules by TLRs, can lead to chronic inflammation and auto-sensitization, thereby promoting or favoring inflammatory and autoimmune diseases [127]. There is increasing evidence indicating that the TLR-mediated pathways play a role in systemic lupus erythematosus [128], type 1 diabetes [129], rheumatoid arthritis [130] and multiple sclerosis [131]. In regard to multiple sclerosis, recently Tzartos and colleagues [132] provided evidence of the activation of TLR3-mediated innate immune response in active lesions of multiple sclerosis brain overexpressing inflammatory IFN- α , where signs of EBV latency were found [132,133] suggesting that EBV-driven innate immune responses might contribute to neuroinflammation in the brain of multiple sclerosis patients.

The contribution of TLRs in autoimmunity can mainly be explained by the ability of these receptors to stimulate maturation of antigen-presenting cells, production of IFN-I and other proinflammatory cytokines, which in turn cause priming of adaptive immune cells, such as autoreactive T-cells [126]. Chronic inflammation is a major feature of target organs in many organ-specific autoimmune diseases and a major contributing factor in the development or maintenance of autoimmunity; it represents a crucial event that leads from TLR activation to auto-sensitization and autoreactivity. Moreover, there is experimental evidence that, along with antigen binding to the B-cell receptor and CD40 stimulation, TLR stimuli, mainly those mediated by TLR3, 7 and 9, provide additional costimulatory signals for proliferation, maturation and survival of B-cells, including autoreactive B cells, thus compromising B-cell tolerance [134–136]. In vitro and in vivo studies suggested that persistent activation of nucleic acid-sensing TLRs (i.e. TLR7, 8 and 9) may cause systemic autoimmunity, through stimulation of B-cell activation and autoantibody production [137]. However, a tolerogenic role for TLR9 has recently been described by Miles and colleagues [138]; they reported evidence that TLR9 engagement in B cells in response to DNA complexes expressed by apoptotic cells is essential for maintaining peripheral B-cell tolerance, thus suggesting that the balance between regulatory and inflammatory function of TLR9 may be crucial for avoiding autoimmunity.

TLR stimuli have recently found to influence regulatory T cell function. In a study of Hackl and collaborators [139], in vitro and in vivo activation of dendritic cells by TLR7 ligands reduced regulatory T cell generation and the suppressive function, suggesting that TLR7

Table 2

TLR expression in the thymus of MG and non-MG patients.

TLR	Ligands	Thymic transcriptome study [79]	PCR on thymus
TLR1	Triacyl lipoproteins from bacteria	Overexpressed only in MG with thymic hyperplasia	nd
TLR2	Microbial molecules from Gram-positive and -negative bacteria, mycoplasma and yeast	Overexpressed in MG	Down regulated in MG [133] Overexpressed in MG (Le Panse, UD)
TLR3	dsRNA from viruses	Overexpressed in MG	Down regulated in MG [133] Overexpressed in MG [63]
TLR4	Lipopolysaccharide from Gram-negative bacteria	nd	Overexpressed in MG [133]
TLR5	Flagellin from Gram-positive and -negative bacteria	No significant change	No significant change [133]
TLR6	Diacyl lipoproteins from bacteria and mycoplasma	nd	nd
TLR7	ssRNA from viruses	No significant change	Overexpressed in MG (Cavalcante, UD)
TLR8	ssRNA from viruses	nd	Overexpressed in MG (Le Panse, UD)
TLR9	Unmethylated CpG oligonucleotide DNA from viruses and bacteria	nd	Overexpressed in MG (Cavalcante, UD)
TLR10	Unknown	Overexpressed in MG	nd

nd = not done; UD = unpublished data.

activation in response to viral infections may compromise tolerance maintenance by regulatory T cells.

In view of the “pro-autoimmunity” effect of TLRs, therapies targeting TLR pathways, mainly based on the use of TLR antagonists, are currently testing in clinical trials for both systemic and organ-specific autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis [131].

4.2. TLR activation in MG thymus

The observation of IFN-I and IFN-III overexpression in MG thymus suggests a link of MG to pathogen infection. If numerous studies suggest that TLR activation could be involved in various autoimmune diseases [140,141], few data are available on MG. A recent paper analyzed TLR mRNA expression in PBMCs from MG and non-MG patients, observing in MG a decreased expression for TLR1, TLR6 and TLR10 and an increased expression for TLR2, TLR3, TLR4, TLR5, TLR8, and TLR9. In addition, they found that TLR9 mRNA expression is significantly related to the clinical severity of the disease which suggests that TLR signaling may contribute to MG pathogenesis [142]. The thymus is a common target organ for infectious diseases [143]. Changes regarding TLR expression in MG thymus have been investigated for TLR2, TLR3, TLR4 and TLR5 and a clear increased expression of TLR4 has been observed in the hyperplastic thymus of MG patients [144]. In Table 2, we report the data of TLR mRNA expression in the thymus of MG patients, collected from two MG populations in France and Italy. Different results were obtained regarding TLR2 and TLR3 expression. This might be related to a difference in the level of expression of TLR2 and TLR3 in the control groups (non-MG patients): in the Italian study control thymuses were from babies, whereas in the French study they were from adults. Our unpublished data showed that TLR2 and TLR3 mRNA levels were higher in the thymus of babies than in adults. It is also possible that the observed discrepancy may be due to exposure to different pathogens in the two countries.

Recently, to investigate the potential consequences of pathogen infection on thymus, we analyzed the effects of TLR agonists (from TLR1 to TLR9) on human thymic epithelial cell cultures. We demonstrated that poly(I:C), a synthetic analog of dsRNA, specifically induces thymic overexpression of α -AChR but no other AChR subunits or tissue-specific antigens. This induction is mediated through TLR3 and protein kinase R (PKR), and by the release of IFN- β . Interestingly, we also showed the overexpression in the MG thymus of TLR3, PKR, IRF7, IRF5 and IFN- β that are all involved in antiviral responses to dsRNA [73]. In addition, poly(I:C) injections in wild-type mice, but not in IFN-I receptor knockout mice, specifically increased thymic expression of α -AChR and, at later time points, induced an anti-AChR autoimmune response characterized by a significant production of serum anti-AChR antibodies, a specific proliferation of B cells and

MG-like clinical signs [73]. This study makes a link between a potential viral etiological mechanism in MG and the specific immune response to AChR.

4.3. Role of viral infections in thymic MG pathogenesis

As for other autoimmune diseases, the contribution of pathogen infections to MG pathogenesis has long been suspected. Several mechanisms have been postulated to explain how pathogen infections might trigger autoimmunity [78]. Based on the early theory of “molecular mimicry” [145], that was documented in many animal models of autoimmune diseases, autoreactivity could be the result of an immune response to microbial antigens that is able to activate T-cells crossreacting with self antigens [78]. Another possible mechanism related to infection-induced autoimmunity is referred to as the “bystander effect” that occurs secondary to infection. The initial viral infection induces a localized inflammation of the target organ and triggers an autoantigen sensitization [79]. In recent years, accumulating data indicate that the general activation of the immune system by innate immune components responding to pathogens may be a pivotal mechanism linking infections to autoimmunity. In that way, B-cell activation by infectious agents, such as EBV, bacterial DNA or CpG sequences may also contribute to autoimmunity initiation [134].

Although infectious agents have not been unequivocally demonstrated as triggering factors in MG, some “early” and more recent data, strongly support the idea that pathogens could significantly contribute to MG development and progression. Cases of MG with disease onset occurring immediately after proven infection with measles [146], EBV [147], or co-infection with human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV) [148], have been documented. The observation that cross-reactivity may exist between AChR peptides and proteins from herpes simplex [149], *Escherichia coli*, *Proteus vulgaris*, and *Klebsiella pneumoniae* [150] suggested that molecular mimicry could be a mechanism implicated in virus-mediated autoimmunity in MG. Early serological studies aimed at identifying antiviral antibodies in MG serum and trying to correlate viral infections with the disease, produced contrasting results [151,152]. However, a recent study of Csuka and collaborators [153] showed that high levels of EBV nuclear antigen (EBNA)-1 antibodies are more common in MG than healthy controls and are particularly associated with EOMG, supporting the hypothesis that, similarly to multiple sclerosis [154], an association between EBV infection and EOMG might exist, highlighting the need to further investigate the role of EBV in EOMG.

Some early attempts to detect or isolate viruses from homogenates or cell suspensions from MG thymuses failed [155,156], but the negative results could well be due to the low sensitivity of the detection techniques or to the procedures of storing and processing the

thymus tissues, that may not have been optimal compared with those used today. Nevertheless, a study of McGuire and colleagues [157] provided evidence of EBV DNA in thymuses of 2 out of 4 MG patients with thymic hyperplasia and 2 out of 2 patients with thymoma, suggesting a role of EBV in autoimmunity and thymoma development in MG. The inflammatory signature of hyperplastic MG thymus [91] and the increased expression of TLR3 and TLR4 that we observed in some MG thymuses [73,144] are compatible with the hypothesis that viral infections could underlie the intra-thymic MG pathogenesis. By using PCR-based techniques, we investigated the presence of cytomegalovirus, herpes zoster virus, herpes simplex types 1 and 2, eubacteria, respiratory syncytial virus, and enteroviruses – viruses known to activate TLR4 – in MG thymuses positive to TLR4 and identified the presence of poliovirus type 1 (PV) RNA in 4 out of 27 (14.8%) of MG tissues examined [158]. None of 18 non-myasthenic control thymuses examined was positive to PV RNA. In PV positive cases, we detected plus- and minus-strand PV RNAs and found a linear correlation between their levels, thus indicating persistent PV infection. Immunolocalization of the viral capsid protein of enteroviruses in PV RNA positive MG thymuses revealed the presence of PV in macrophages expressing TLR4, that were disseminated in the thymic parenchyma. The finding of a persistent PV infection in the thymus of some MG patients strongly supported the idea that, in the context of a genetic background predisposing to MG, a persistent or dysregulated TLR4-mediated innate immune response to pathogens invading the thymus could cause or sustain thymic chronic inflammation, thus favoring autosensitization and autoimmunity (Fig. 1). Recently, we provided another important finding strengthening the concept of a viral contribution to MG etiology: the identification of an active EBV infection as dominant features of the thymus of EOMG patients [159]. EBV, a widely diffused B-lymphotropic gamma-herpes virus that infects most of the world population, has been associated with the etiology of several autoimmune diseases, like multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis [160]. Similarly to the brain of multiple sclerosis patients, in which Serafini and co-workers found multiple signs of active EBV infection [133], we provided evidence for the first time of EBV persistence and reactivation in the thymus of EOMG patients, but not in control thymuses from adult healthy subjects [159,161]. This finding supported the idea of a possible common mechanism involving EBV to explain persistent inflammation and autoimmunity perpetuation in autoimmune conditions, as multiple sclerosis and MG, characterized by B-cell activation, lymphoid neogenesis and chronic inflammatory status in the target/affected organs. Thymic EBV signature could also explain dsRNA-signaling pathway activation observed in MG thymus [73]. Indeed, EBV encodes small RNAs (EBER) that trigger TLR3 signaling and induce IFN-I and proinflammatory cytokine expression, similarly to poly(I:C), a synthetic dsRNA [162]. The ability of EBV to infect and transform B-cells, allowing them to evade immune surveillance [160], makes it a prime suspect for chronic infection and inflammation. The concentration of the virus in MG thymus could provide an explanation for how the autoimmune response can be perpetuated in this organ in MG patients, since EBV could activate and immortalize intra-thymic autoantibody-producing cells and sustain chronic inflammation via TLR-mediated pathways [163]. The recruitment of peripheral lymphoid cells in the thymus by inflammatory chemokines could create a “vicious circle” in which new events of autosensitization and EBV infection could sustain and perpetuate the thymic autoimmune response. Whether a pre-existing inflammatory state, of different origin than EBV infection, is responsible for the recruitment of EBV-harboring cells in MG thymus and for subsequent EBV reactivation, is unknown. In contrast with our findings, two recent studies reported absence or very low levels of EBV infection in the thymus of EOMG patients [164,165]. Different tissue processing and different procedures to detect EBV were used and this could explain the discrepancies in the obtained results [166]. Since EBV search in human tissues may be

extremely challenging, technical issues related to tissue storage and processing, and use of more or less sensitive techniques, could be critical to obtain successful results. The use of a combination of techniques, as in situ hybridization, immunohistochemistry and molecular techniques to detect various EBV markers [159,161], applied on optimal stored and processed tissues and with the use of appropriate positive and negative controls, could be a correct approach to further investigate EBV presence in the thymus, as well as in other tissue types. Given the potential therapeutic implications that could have EBV confirmation as pathologic feature of MG thymus, additional studies investigating more deeply the EBV relationship with MG are worthy to be done to provide new insights.

5. Conclusions

MG is a complex autoantibody-mediated neurological disease involving both genetic and environmental risk factors. Different MG subtypes, usually defined by autoantibody signature, disease severity, age at onset and thymic involvement, exist and could differ in etiology. AChR-MG occurring in EOMG patients and presenting with thymic hyperplasia is the most common, and also the best characterized, subtype of MG. An intra-thymic pathogenic link between innate immunity and autoimmunity can be postulated for AChR-MG: the observation of an IFN and TLR signature in the hyperplastic MG thymus, along with data showing that inflammatory cytokines can increase the expression levels of α -AChR in thymic epithelial cells and myoid cells, suggest the hypothesis that, in the context of a genetic predisposing background, intra-thymic infection events might contribute to the specific immune response to AChR, by eliciting altered innate immune responses that lead to anti-AChR autosensitization and adaptive autoimmune response (Fig. 1). The fact that dsRNA-signaling activation can induce thymic changes related to MG and even trigger a specific anti-AChR response in mice speaks for a viral etiological mechanism in MG. Moreover, our recent finding of an active EBV infection in B-cells/plasma cells of MG thymus provides an explanation on how the autoimmune response can be perpetuated in this organ, since EBV is potentially able to immortalize thymic autoreactive B cells that then can leave the thymus and perpetuate autoreactivity in periphery. EBV itself could sustain, alone or together with other pathogens (co-infection events), chronic inflammation and dysregulated TLR activation in MG thymus. Further investigation is needed to dissect the mechanisms postulated in our proposed model.

Take-home messages

- Variation in autoantibody status is associated with different MG clinical phenotypes.
- HLA loci are the main genetic factors implicated in MG susceptibility.
- The thymus is the prime site of autosensitization to AChR in MG.
- TLR-mediated innate immune response is involved in the thymic etiology of MG.
- Active EBV infection was found in the thymus of MG patients.

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Conflicts of interest

P.C., P.C., R.L.P and S.B.A have no conflict of interest.

R.M. has served on a scientific advisory board for GlaxoSmithKline, Alexion, and Bio-Marin and has received funding for travel and

Fig. 1. Proposed model of pathogenic gene-environment interactions in AChR-MG etiology. Genetic risk factors, mainly HLA risk haplotypes and variants in the gene encoding the α -subunit of AChR (CHRNA1), predispose to AChR-MG by altering AChR presentation/cross-presentation and causing defective immunoregulatory mechanisms. Possible genetic variants in TLR genes (not yet investigated) might predispose to altered (dysregulated, persistent) innate immune response to infections. In this genetic context, colonization of the thymus by pathogens is crucial for the development of the anti-AChR autoreactivity. Intra-thymic persisting viruses (as EBV and other pathogens, as single or co-infection) activate dysregulated TLR-mediated signaling pathways that in turn cause chronic inflammation and anti-AChR auto-sensitization, as a result of: TLR-dependent activation of antigen-presenting cells; increased expression of the autoantigen on thymic epithelial cells or myoid cells; increased autoantigen cross-presentation; and, finally, activation of auto-reactive T-cells, that promote autoimmune response by autoreactive B cells. EBV is able to promote the maintenance of the autoimmune response within the thymus, by immortalizing autoreactive B cells. Other environmental factors, such as vitamin D deficiency, drugs and hormones can contribute to intra-thymic autoimmunity by altering thymopoiesis and immune cell function, thus favoring central tolerance disruption.

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Anti-ganglioside antibodies are not useful as a serological marker of neuropsychiatric involvement in patients with systemic lupus erythematosus [USED]

Anti-ganglioside antibodies (AGA) have been proposed as putative serological markers of neuropsychiatric systemic lupus erythematosus (SLE), but recent findings are controversial. These autoantibodies are involved in the pathogenesis of several peripheral immune-mediated neuropathies. In order to investigate the potential role of AGA in neuropsychiatric SLE, Labrador-Horrillo et al. (*Lupus* 2012;21:611–5) tested the presence of AGA in the sera of a large cohort of consecutive SLE patients with or without active neurological involvement according to the 1999 ACR criteria for neuropsychiatric lupus syndromes. IgG or IgM AGA specific for different ganglioside antigens were detected by standard ELISA and confirmed by thin layer chromatography. AGA, mainly of the IgM isotype and specific for GM1 ganglioside, were exclusively found in about 30% of SLE patients with neuropsychiatric involvement, but they did not correlate with any neurological manifestation in particular. Thus, the authors concluded that serum AGA are not useful as biomarkers of neurological complications in SLE patients.

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