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Chapter

Epstein-Barr Virus in Myasthenia Gravis: Key Contributing Factor Linking Innate Immunity with B-Cell-Mediated Autoimmunity

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Abstract

Epstein-Barr virus (EBV), a common human herpes virus latently infecting most of the world's population with periodic reactivations, is the main environmental factor suspected to trigger and/or sustain autoimmunity by its ability to disrupt B-cell tolerance checkpoints. Myasthenia gravis (MG) is a prototypic autoimmune disorder, mostly caused by autoantibodies to acetylcholine receptor (AChR) of the neuromuscular junction, which cause muscle weakness and fatigability. Most patients display hyperplastic thymus, characterized by ectopic germinal center formation, chronic inflammation, exacerbated Toll-like receptor activation, and abnormal B-cell activation. After an overview on MG clinical features and intra-thymic pathogenesis, in the present chapter, we describe our main findings on EBV presence in MG thymuses, including hyperplastic and thymoma thymuses, in relationship with innate immunity activation and data from other autoimmune conditions. Our overall data strongly indicate a critical contribution of EBV to innate immune dysregulation and sustained B-cell-mediated autoimmune response in the pathological thymus of MG patients.

Keywords: autoimmunity, Epstein-Barr virus, innate immunity, myasthenia gravis, thymus, toll-like receptors

1. Introduction

Myasthenia gravis (MG) is a clinically heterogeneous, B-cell-mediated disorder affecting the neuromuscular junction (NMJ) and is mostly caused by the abnormal production of autoantibodies against the acetylcholine receptor (AChR) located in the postsynaptic membrane [1]. MG is generally considered a prototypical autoimmune disease, since the main target of the autoimmune response in the affected patients is well known. However, it is uniquely characterized by morphological and functional thymic abnormalities (hyperplasia and thymoma), which make this organ the main site of immunological alterations leading to the disease. Indeed, if the muscle is the target organ in MG patients, the thymus is now widely accepted as the main effector organ, in which B-cell expansion, anti-AChR autosensitization, and autoimmune response arise and are perpetuated [2]. The exact mechanisms

triggering and sustaining autoimmunity in MG thymus are still unknown. As with many autoimmune conditions, considerable evidence indicates a multifactorial MG pathogenesis based on complex interactions among multiple genetic and environmental factors and their interplay with the immune system [3]. Among environmental factors, viruses are the main suspects to play a role in autoimmune diseases, mainly by their ability to induce persistent or aberrant Toll-like receptor (TLR)-mediated innate immune responses, which are able to promote or favor autoimmunity in genetically susceptible individuals. In our studies, we provided evidence of dysregulated Epstein-Barr virus (EBV) infection in hyperplastic and thymomatous MG thymuses in association with TLR overexpression, thus revealing EBV as a key contributing factor to intra-thymic B-cell tolerance disruption and sustained B-cell-mediated autoimmunity in MG patients [4].

2. Myasthenia gravis: autoantibodies and clinical features

MG is an autoimmune disorder of the NMJ, leading to fluctuating weakness and fatigability of skeletal muscles, exacerbated by repetitive contraction and improved on resting. Frequently, MG starts with ocular symptoms, as diplopia and ptosis, but in 80–85% of cases, ocular disease progresses to a generalized form within the first 2–5 years from onset, involving skeletal, bulbar, or respiratory muscles [5]. Respiratory failure (myasthenic crisis) occurs in 15–20% of patients and can be observed in younger and older patients [6].

MG is a heterogeneous condition whose clinical variability allows classification of patients in distinct disease subgroups, mainly based on autoantibodies, age at onset, and thymic histology [1, 7, 8]. In more than 80% of patients, the autoimmune attack is mediated by autoantibodies against AChR, less frequently (1–5%) against the muscle-specific kinase receptor (MuSK) or the low-density lipoprotein receptor-related protein 4 (LRP4), two proteins involved in AChR clustering. In addition, autoantibodies against other NMJ components, including cortactin, agrin, titin, and ryanodine receptor (RyR), have been described, especially in late-onset or thymoma-associated disease; their presence is concomitant to anti-AChR autoantibodies and indicates more severe manifestations [7]. Around 10% of generalized (non-ocular) patients results negative for anti-AChR, anti-MuSK, or anti-LRP4 antibodies. Clinically, these seronegative patients are similar to AChR-MG patients and can show thymic hyperplastic changes [9]. The triple seronegative MG subgroup may be heterogeneous, including patients with antibodies having affinities or concentrations too low to be detected with standard routine assays, or patients with antibodies against relevant antigens not identified yet. The introduction of cell-based assays (CBAs), having increased sensitivity compared to the routine assays commonly used, has significantly increasing the chance to identify autoantibodies to low-affinity clustered AChR, MuSK, and LRP4, thus improving MG diagnosis [7, 10–12].

As mentioned above, AChR-MG is associated with thymic patho-histological changes, including follicular hyperplasia and thymoma [13, 14]. Its severity is related with the loss of AChRs on NMJ, but not with the titers of circulating autoantibodies [15]. According with age at onset, AChR-MG follows a bimodal pattern, with the first peak under 50 years (early-onset MG, EOMG), and a second peak >50 years (late-onset MG, LOMG) [16, 17]. EOMG occurs most in young women and is generally associated with thymic hyperplasia. LOMG, which usually is generalized, mainly affects men, who frequently present thymic involution. Thymoma-associated MG that presents more severe symptoms can occur at any age, though it is more frequent in the elderly myasthenic patients and is frequently associated with the presence of antibodies against RyR and titin along with anti-AChR antibodies [14, 17].

MuSK-MG patients are mainly young females and typically have severe clinical symptoms [18]. An intra-thymic pathogenesis for MuSK-MG is not considered relevant, although a recent study found hyperplasia in 23% of MG patients positive for anti-MuSK antibodies by CBA [11]. LRP4-MG is less characterized but largely overlaps with AChR-MG clinical features; typical thymic histopathology has been recorded with a sparing of thymoma, at least in the European multinational cooperative study by Zisimopoulou and colleagues [12]. Most of LRP4-MG patients present ocular or generalized mild manifestations, and about 20% have only ocular weakness for more than 2 years [12, 19].

In MG, degradation of the postsynaptic membrane results in decreased AChRs and voltage-gated sodium channels, causing a significant reduction of endplate potential and raising the firing threshold, which is required to generate an action potential. Thus, during prolonged synaptic activity, as the quantal ACh content normally runs down, the summation of endplate potentials falls below the threshold, and they can no longer trigger the action potential of muscle fibers, leading to typical muscle weakness [20]. Three mechanisms of action of anti-AChR autoantibodies can explain NMJ impairment: (1) functional AChR block due to autoantibodies binding to the ACh-binding sites; (2) cross-linking and subsequent AChR internalization due to the ability of autoantibodies to target two antigen molecules (antigenic modulation); and (3) complement pathway activation, which leads to the generation of the membrane attack complex (MAC) and hence the destruction of the postsynaptic membrane. Complement activation at NMJ is thought to be the main pathogenic mechanism of anti-AChR antibodies, which are mainly of the IgG1 and IgG3 classes and therefore can bind and activate the complement system [20].

MuSK antibodies are generally IgG4, lacking complement-fixing, and are considered functionally monovalent, being unable to induce antigenic modulation. They affect MuSK ability to maintain the correct AChR cluster at the NMJ, by inhibiting the formation of MuSK-LRP4 complex and the agrin-stimulated MuSK phosphorylation [21, 22]. In addition, anti-MuSK antibodies are able to block binding of ColQ to the NMJ, compromising agrin-mediated AChR clustering [23]. Experimentally, animals receiving repeated daily injections of MuSK-positive patients' IgG, or actively immunized with MuSK, show impaired neuromuscular transmission, with reductions in endplate AChRs [24].

Both AChR-MG and MuSK-MG fulfill Witebsky's criteria for autoimmune diseases [25]. LRP4-MG also seems to adhere to these criteria: mice immunized with the LRP4 extracellular domain, or with IgGs purified from LRP4-immunized rabbits, present anti-LRP4 antibodies, exhibit MG-associated symptoms and their serum is able to decrease cell surface LRP4 levels [26, 27]. Anti-LRP4 autoantibodies mainly belong to the IgG1 subclass, thus they can activate the complement system; moreover, they prevent agrin-induced MuSK activation and AChR clustering [27].

Current therapeutic approaches for MG include symptomatic treatment with cholinesterase inhibitors, non-specific immunosuppression with corticosteroids and thymectomy in selected patients. Plasmapheresis or immunoglobulins are used for acute management of severe muscular weakness [8]. New biological drugs targeting molecules involved in the specific immune-pathological mechanisms, like eculizumab, which blocks the C5 terminal complement component, and efgartigimod, a functional IgG neonatal Fc receptor blocker, are promising for more specific and effective intervention to reduce corticosteroids side effects and to treat refractory patients [8].

3. Role of the thymus in MG pathology

The thymus is a primary lymphoid organ that provides a complex environment essential for T-cell differentiation and the establishment of central tolerance. It

is composed of various cell types, mainly thymocytes and thymic epithelial cells (TECs), but also myoid cells, dendritic cells (DCs), macrophages, and B-cells in a limited number. By expressing tissue-specific antigens, mainly via the transcription factor autoimmune regulator (AIRE), medullary TECs play a key role in negative selection of thymocytes. Indeed, interactions between these cells and developing thymocytes lead to the elimination of autoreactive T cells, whereas the self-tolerant T cells continue their differentiation through the different thymus compartments, to be exported to the periphery [28].

Structural and functional pathological alterations of the thymus are found in approximately 80% of AChR-MG patients with generalized disease, including thymic hyperplasia (about 70% of patients) and thymoma (10–15%); the remaining patients (10–20%) present an atrophic or involuted thymus, mainly consisting of adipose tissue with residual areas of thymic parenchyma, in some cases showing hyperplastic changes (**Figure 1**) [13, 29, 30]. Hyperplasia is characterized by the
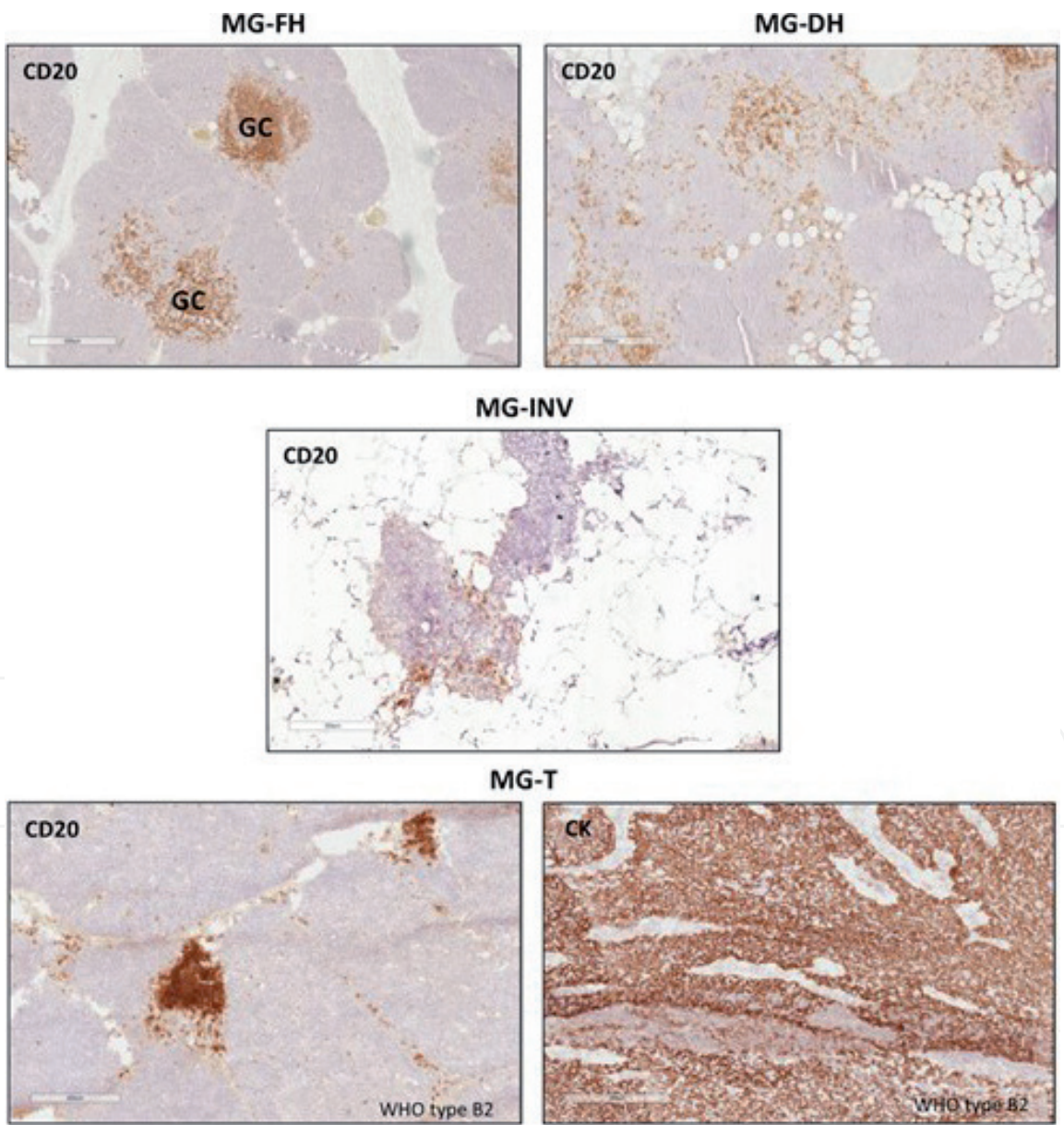
Figure 1.
Pathological abnormalities of myasthenia gravis thymus. Immunohistochemistry stainings showing CD20-positive B-cells in ectopic germinal centers (GCs) and lymphoid infiltrates of follicular hyperplastic (MG-FH) and diffuse hypeplastic (MG-DH) thymuses. CD20-positive B-cells are also scattered throughout the residual thymic parenchima in involuted MG thymuses characterized by abundant interlobular fat (MG-INV). CD20-positive B-cells, isolated or present as aggregates, can be detected in the cytokeratin (CK)-positive tumoral tissue of MG thymomas (MG-T).

presence of B-lymphocyte infiltrates invading the thymic medulla, or present in expanded perivascular spaces fused with the thymic medulla. B-cell infiltrates can be scattered throughout the medullary parenchyma (diffuse hyperplasia) or be organized into ectopic B-cell germinal centers (GCs) which, together with DCs and follicular helper T (Tfh) cells, form follicles (follicular hyperplasia) [30]. GCs are microarchitectures specialized to produce high-affinity antibodies against antigens, to establish the humoral immune response [31]. They are present in secondary lymphoid organs but rarely into the thymus, implying that GC development in the thymus of MG patients is a pathological event related to autoimmunity development. Ectopic GC formation is a characteristic feature of other organs target of chronic inflammation and autoimmunity, including multiple sclerosis (MS) brain and synovia of rheumatoid arthritis (RA) patients, indicating that lymphoid neogenesis plays a relevant role in the immune-pathological process of inflammatory autoimmune conditions [31–33]. In MG, GC formation is associated with the production of high endothelial venules expressing inflammatory chemokines, that abnormally recruit peripheral immune cells into the thymus, including the chemokine ligand 21 (CCL21), a key molecule that orchestrates thymic hyperplastic changes by promoting B-cell infiltration [34]. Uniquely, MG thymic GCs are surrounded by muscle-like myoid cells expressing AChR and other muscle antigens, along with plasma cells [35], thus supporting the idea that GCs may be the site of autosensitization and autoantibody production in the thymus of MG patients.

A wealth of data indicates that MG thymus contains all the elements necessary for developing and perpetuating an AChR-specific autoimmune response: TECs and muscle-like myoid cells expressing the autoantigen, professional antigen-presenting cells, AChR-specific autoreactive T cells and B-cells, and plasma cells producing autoantibodies [30]. Indeed, transplantation of MG thymic fragments to immunodeficient mice induces the formation of anti-AChR antibodies and their deposition at the skeletal muscle endplates [36].

As regard to the autoantigen presentation, TECs express major histocompatibility (MHC) class II complex and AChR subunits, including α, β, and γ subunits [37]. Thymic myoid cells express not only AChR subunits but also a functional AChR, whose fragments can be presented to T lymphocytes by cross-presentation via DCs, since myoid cells do not express MHC class II molecules [38, 39]. Cross-presentation may be favored by a persistent autoantibody and complement-mediated attack to myoid cells, which make the levels of autoantigen more available to DCs [40, 41]. Both TECs and myoid cells respond to pro-inflammatory cytokines by increasing the expression of AChR components, mainly the α subunit, which contains the main immunogenic region, thus suggesting that inflammation in the thymic microenvironment can results in enhanced autoantigen presentation and possible autosensitization against AChR [42]. Indeed, several lines of evidence indicate that MG thymus is in a state of chronic inflammation, characterized by an overexpression of pro-inflammatory cytokines and chemokines (IL-6, CCL19, CCL21, CXCL10, CXCL11, CXCL13, and RANTES) [43]. Among cytokines, type I interferons (IFNs) and IFN-induced genes are significantly up-regulated in hyperplastic MG thymuses and have been critically involved in driving thymic events that can lead to follicular hyperplastic changes, AChR overexpression and autosensitization. In particular, IFN-β was found to increase α-AChR-specific expression in TECs, along with the expression of inflammatory chemokines (e.g., CXCL13 and CCL21), and was able to recruit T and B-cells into the thymus, as well as B-cell activating factor (BAFF), which favors B-cell survival [44]. In hyperplastic MG thymus, no changes in the frequency of CD4+ and CD8+ T cells exported to the periphery was observed, but functional defects of regulatory T cells (Tregs) were demonstrated, along with resistance of conventional T cells to the Treg immunosuppressive function, thus indicating dysregulation

of immunoregulatory mechanisms [45]. Altered Treg/T effector cell balance was associated with increased expression of pro-inflammatory cytokines by MG T cells, mainly IL-17, IFN-γ, IL-21, and tumor necrosis factor α (TNF-α) [45].

Thymoma is a rare thymic epithelial tumor, associated with autoimmune and paraneoplastic syndromes. The most common thymoma-associated autoimmune disorder is MG: up to 50% of thymoma cases may develop MG, whereas 10–15% of MG patients present thymoma [46]. Histologically, thymoma is a slow growing, locally invasive tumor consisting of transformed epithelial cells surrounded by maturing polyclonal T cells. The most recent World Health Organization (WHO) classification identified five types, A, AB, B1, B2, and B3, based on the nature of the cortical or medullary epithelial cells, and on the proportion of lymphocytes, with B2 and AB being the WHO types most frequently associated with MG [47, 48]. MG associated with thymoma usually has worse prognosis, showing generalized and maximum severe symptoms [48]. Alterations typical of the thymoma microenvironment may explain the development of AChR-specific autoimmunity in thymoma patients. They include: the lack of a functional medulla expressing AIRE; the reduction, or absence, of tolerogenic myoid cells; the reduced expression of HLA class II molecules; and the failure of Treg generation, which ultimately leads to autosensitization to AChR, and other locally expressed muscle antigens, and defective negative T-cell selection [14, 49, 50]. Recently, a higher proportion of GCs was abnormally found in non-neoplastic thymic tissue adjacent to thymoma in MG patients compared to thymoma patients without MG, suggesting that B-cell dysregulation characterizes not only thymic hyperplasia but also thymoma-associated MG, and that GCs may represent a risk factor for the development of MG in thymoma patients [51].

An important evidence of the central contribution of the thymus in autoimmune response development and maintenance in MG is the ability of thymectomy to improve the disease course in non-thymomatous patients over a 2-year period after the surgery, as demonstrated by the MGTX clinical trial, and its extension study [29, 52]. Thymectomy is mandatory for thymoma patients. Its efficacy as treatment option in non-thymomatous patients is plausibly due to eradication of the site of autoimmunity, as indicated by the fact that AChR antibody titers usually fall after thymectomy, and the magnitude of this fall correlates with the proportion of GC B-cells in the removed thymus [53]. However, autoantibody titers do not always fall, suggesting other possible sites of autoantibody production in some patients [54].

3.1 Innate immunity and toll-like receptors: a pathogenic link with autoimmunity

Innate immunity is the first line of defense against pathogen infections. Its interplay with the adaptive-humoral immune system plays a key role in central and peripheral tolerance maintenance, and strictly depends on a fine regulation of TLRs. TLRs are a family of pattern recognition receptors (PRRs) able to recognize specific conserved microbial-derived molecular structures, thus sensing danger signals. They are expressed by a variety of cell types, but mainly by innate immune cells, such as DCs and macrophages [55]. The TLR family includes at least 11 members in humans: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, which are located on the cell surface and recognize microbial membrane-associated molecules (e.g., LPS, lipoprotein, and peptidoglycan); TLR3, TLR7, TLR8, TLR9, and TLR11, present on the intra-cellular endosome membranes and able to distinguish bacterial or viral nucleic acids, including ssRNA, dsRNA, and unmethylated cytosine phosphate guanine (CpG)-containing DNA [55]. Upon its own specific ligand binding, TLR dimerizes, internalizes, and interacts with the intracellular adaptor myeloid differentiation primary response gene 88 (Myd88) or with the TIR

domain-containing adapter-inducing IFN-β (TRIF), which activates nuclear factor κB (NFκB) and IFN response factor transcription. The TLR signaling cascade induces the expression of pro-inflammatory agents (e.g., IFN-I, IL-6, IL-12, IL-23, and TNF-α), which in turn contribute to activate immune system cells [56]. In this way, the first function of TLRs is to set up an innate immune response to protect the organism from pathogens. However, TLRs have been implicated in several autoimmune diseases, including systemic lupus erythematosus (SLE), RA, and MS, by studies performed in humans and animal models [56, 57]. Specifically, dysregulated or persistent TLR activation has been demonstrated to contribute to autoimmunity by (i) abnormal stimulation of antigen-presenting cell maturation and increased IFN-I and pro-inflammatory cytokine production; (ii) altered balance between Treg and T helper 17 (Th17) cells; (iii) induction of co-stimulatory signals for proliferation, maturation, and survival of B-cells, which compromise B-cell tolerance; and (iv) promotion of GC formation [56–58].

In hyperplastic MG thymuses, overexpression of TLR3, TLR4, TLR7, and TLR9, in association with chronic inflammation, has been demonstrated in different studies, thus supporting the existence of a critical cross talk between innate immunity and autoimmunity in the intra-thymic MG pathogenesis [59–63].

Our group was the first to demonstrate a marked overexpression of TLR4 in involuted and hyperplastic MG thymuses, especially in TECs [59]. Later, we revealed that TLR4 stimulation in MG TECs was able to increase the production of Th17-related cytokines and the expression of CCL17 and CCL22, two chemokines involved in peripheral immune system cell recruitment in inflamed organs, that we found to be overexpressed in MG thymuses [60]. Moreover, by generating an in vitro imaging model based on experimental autoimmune MG co-cultures of Th1/Th17 AChR-specific T cells, naïve Tregs, DCs, and TECs, we found that TLR4 stimulation increased AChR-specific T-cell activation and impaired Treg function, thus disclosing a contribution of dysregulated TLR4 signaling to the inflammatory and autoimmune process in the MG thymic milieu [60].

Cufi and colleagues showed that also TLR3 is overexpressed in MG thymuses and demonstrated that stimulation of this TLR with its ligand poly(I:C), a synthetic analog of viral dsRNA, induced a specific up-regulation of α-AChR in TECs but not of other AChR subunits or tissue-specific antigens, via IFN-β release [61]. Of interest, another study of the same group disclosed that poly(I:C) injection in wild-type mice, in combination with LPS, that stimulates TLR4 was able to increase α-AChR thymic expression and induce thymic hyperplastic changes along with production of serum anti-AChR antibodies [62]. These mice developed MG symptoms in absence of any AChR immunization, thus supporting the idea that pathogen infections could contribute to anti-AChR sensitization and autoimmunity development via persistent or abnormal TLR stimulation [62].

Along with TLR3 and TLR4, TLR7 and TLR9 have also been implicated in the intra-thymic pathological events leading to MG. Indeed, we recently revealed a significant TLR7 and TLR9 up-regulation in both involuted and hyperplastic MG thymuses compared to normal thymuses, with the two receptors being mainly expressed in B-cells, TECs, plasmacytoid DCs, and macrophages [63]. TLR7 was also enhanced in thymic myeloid DCs and its transcriptional levels positively correlated with those of IFN-β [63]. Interestingly, as described in the following paragraphs, the two receptors were markedly expressed in B-cells and plasma cells positive for EBV proteins, indicating EBV as contributing environmental factor implicated in dysregulated TLR activation in MG thymuses.

Of interest, due to the key contribution of TLRs to chronic inflammation and immune system dysregulation, their pathways are rapidly emerging as attractive targets for therapeutic strategies able to mitigate or inhibit autoimmune processes [64].

4. EBV as contributing factor to autoimmunity

EBV is the main virus suspected to play a role in autoimmune diseases due to its unique ability to infect, activate, and immortalize B-cells, allowing them to evade immune surveillance, at the same time, promoting inflammatory state via TLR-mediated innate immune responses [65, 66].

EBV is a DNA virus of the herpes virus family transmitted through saliva exchange and infecting approximately 95% of the world's population [65]. EBV primary infection mostly occurs during childhood and shows mild symptoms or more frequently none. However, in adolescence or adulthood, EBV causes infectious mononucleosis in 30–70% of cases, with up to 20% of B-cells being infected [67]. After resolution of primary infection, EBV persists lifelong in the host in rare circulating memory B-cells. In the latent state, it is not detectable by immune system and its genome circularizes and replicates together with the host's chromosomal DNA, resulting in a restricted expression of a maximum of nine viral genes: the EBV nuclear antigens (EBNA1, −2, −3A, -3B, and -3C), the leader protein (LP), and the latent membrane proteins (LMP1, −2A, and -2B). Different expression patterns of EBV latent genes determine the occurrence of EBV latency types I, II, or III, each type being associated with distinct EBV-related diseases [65, 68–70]. EBV-encoded small nuclear RNA (EBER) 1 and 2 as well as EBNA1 are expressed in all the latency types [65]. EBNA2 is expressed during latency type III (known as growth program), typical of newly infected naïve B-cells, lymphoproliferative disorders, and mononucleosis [68–71]. LMP1 and LMP2A, key proteins that rescue infected B-cells from apoptosis, act as functional homologs of CD40 and B-cell receptor (BCR) and are expressed during latency III and II (known as default program), with type II being observed in memory B-cells and GC cells, Hodgkin and non-Hodgkin lymphoma, and nasopharyngeal carcinoma [65, 68]. Finally, latency I (known as true latency) is characterized by EBNA1 and EBER expression only; it is typically observed in rare peripheral blood memory B-cells and is associated with Burkitt's lymphoma [65, 68].

The exact mechanism waking up lytic EBV activities is not clear, but it seems to be the result of dynamic interactions between the host's immune response and the infection state. When infected B-cells differentiate into plasma cells, the promoter of early lytic genes can be reactivated, driving expression of numerous proteins involved in viral activities (i.e., BSLF1, BALF2, BBLF4, and BALF5) [65]. Two genes, BZLF1 and BRLF1, which encode viral transcription factors, orchestrate the transition from viral latency to lytic infection [69]. New virions primarily infect B-cells, and the viral entry is mediated by viral gp350 protein binding to CD21 [70].

Uniquely, EBV ensures early mechanisms of immune evasion, such as the inhibition of IFN pathways, through a viral IL-10 homolog, the suppression of cytotoxic T-cell responses and the down-regulation of MHC class I and II expression. Moreover, some of the viral proteins are anti-apoptotic, including the early antigen restricted (EA/R), which is a Bcl2 viral homolog that protects infected B-cells from apoptosis and immortalize them [71]. Several mature EBV miRNAs also contribute to immune system alterations in the host by modulating expression of genes involved in immune recognition, antigen presentation and cellular migration, such as miR-BHRF1–3, which regulates IFN-inducible T-cell-attracting chemokine CXCL-11 expression, or miR-BART20-5p and miR-BART8, which affect the IFN-γ signal transduction pathway [72].

Despite it is innocuous in most cases, EBV has true pathogenic potential due to persistent latent infection with periodic reactivations. Disruption of the virus-host

balance in susceptible individuals can favor autoimmunity or the abovementioned B-cell malignancies [68, 73]. Among autoimmune diseases, EBV has been associated with MS, SLE, and RA by a number of sero-epidemiological and immunological studies [65, 68, 73]. Moreover, EBV persistence and reactivation have been demonstrated in ectopic B-cell follicles detected in MS brains [74], in the Sjögren's Syndrome salivary glands [75], and in synovia from RA patients [76], suggesting that EBV might be a common pathogenic feature of autoimmune conditions characterized by B-cell activation and lymphoid neogenesis.

Several mechanisms have been described to explain how viruses may rise an autoimmune response, including molecular mimicry and immune system general activation via TLRs. Molecular mimicry is due to T-cell Receptor (TCR) and BCR recognition flexibility, so that a microbial peptides, structurally similar to a self-peptide, may trigger the autoimmune response [77]. As regard to EBV, EBV-encoded proteins, such as BZLF1, share regions with the host transcription factors of the fos/jun family and host ankyrin proteins, which anchor the cytoskeleton and regulate host transcription factors, including NF-kB, which is critically involved in the immune response [78]. Quantitative and qualitative differences in CD4+ T-cell response to EBNA1 have been described in MS patients [79], and a small percentage of EBNA1-specific T-cell clones cross-recognize myelin-derived epitopes [80]. A further well-characterized example of molecular mimicry between EBV and host proteins is the similarity of regions of EBNA1 with ribonuclear protein Smith (Sm) antigen or the Ro self-protein in SLE [81]. There are also several examples of molecular mimicry relevant for RA, related to aminoacid motif sharing between HLA-DRB1 and EBV gp110, cytokeratin and type II collagen, and citrullinated human fibrin and a citrullinated EBNA1 form [82].

Another hypothesis of virus-induced autoimmunity is bystander activation, in which viruses act as super-antigens that promote general activation of the immune system; in this context, specialized antigen presenting cells present self-antigens, obtained by inflamed tissue destruction or by the uptake of local dying cells, to autoreactive T cells [77]. In MS, bystander activation may result in central nervous system damage by CD8+ T-cell cytotoxicity and EBER-induced IFN-α production via innate immune response, both described in post-mortem brain tissue from MS patients [74, 83]. EBV can promote inflammation in the infected organs by innate immune system activation, through release of molecules able to activate diverse TLRs and type I IFN production [66, 84]. The EBV envelope protein gp350 stimulates TLR2, whereas EBERs bind TLR3; moreover, EBV RNA and DNA can activate pathways mediated by TLR7 and TLR9 [66]. Thus, EBV ability to stimulate persistent TLR signaling may also contribute to disrupt immune system balance, favoring chronic inflammation and autoimmunity.

5. EBV in the intra-thymic MG pathogenesis

Evidence of chronic inflammation, GC formation, and TLR overexpression in MG thymus, along with data demonstrating a role of TLRs in inducing anti-AChR autoimmunity and MG symptoms in mice, pointed out the idea that MG pathogenesis could be associated with viral infections [43, 59–63].

Based on the hypothesis that active EBV infection may be a common pathogenic event of organs site of autoimmunity, in our previous studies we searched for the expression of EBV markers in the thymus of MG patients. Of interest, we provided the first demonstration of EBV presence in both hyperplastic and thymoma MG thymuses, but not in normal thymuses and non-MG thymomas, obtaining results

indicative of a contribution of the virus to B-cell dysregulation, TLR overexpression and B-cell-mediated autoimmunity in MG [4, 63, 85–87].

Earlier serological studies to associate MG with EBV produced contrasting results. One of them underlined no significant difference in incidence or antibody titers to EBV, cytomegalovirus, herpes simplex type 1 and other virus, in 104 MG patients compared to age-matched healthy controls, weakening the virus hypothesis in MG pathogenesis [88]. More recently, Bhibhatbhan and colleagues reported a case of young woman with abrupt onset of both MuSK-MG and type I diabetes mellitus, following infectious mononucleosis, bringing attention back to EBV in MG [89]. Few years after, according with a possible association between MG and EBV, Csuka and collaborators discovered a strong association between EOMG and high anti-EBNA1 IgG serum concentration in EOMG patients [90]. Despite these results were conflicting, our recent pathological findings, which are deeply described below, allowed us to include MG among the autoimmune diseases critically associated with EBV.
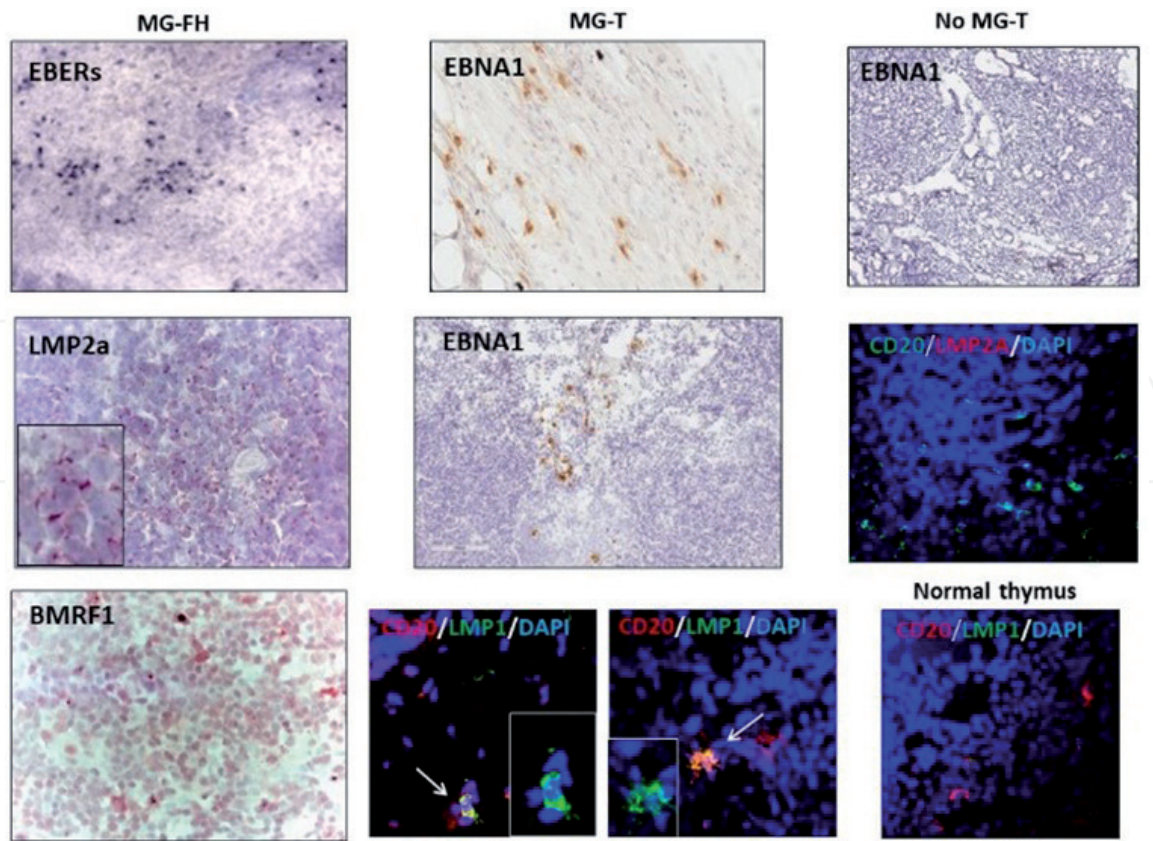
5.1 EBV in hyperplastic MG thymus

To our knowledge, the earliest study attesting EBV presence in MG thymuses was performed by McGuire and colleagues, who found EBV DNA in thymuses of 2/4 MG patients with thymic hyperplasia, and 2/2 patients with thymoma [91]. Before that, several attempts were made for identifying or isolating viruses from homogenates or cell suspensions of MG thymuses, but without success [92, 93]. However, these initial studies used techniques, and tissue storage methods, that cannot be considered sufficiently sensitive or optimal today.

Based on data showing EBV reactivation in intra-meningeal B-cell follicles in MS patients [74], our group decided to check for signs of EBV infection in MG thymuses (n = 17), both hyperplastic (follicular and diffuse) and involuted thymuses by using a combination of techniques, including in situ hybridization (ISH) to detect EBERs, immunohistochemistry (IHC) for latent and lytic EBV antigens, real-time PCR for viral DNA (LMP1 gene), and nested PCR for viral transcripts. As controls, normal thymuses (n = 6) from adult cardiopathic donors without autoimmune diseases were analyzed [85]. Interestingly, all MG, but not normal thymuses, resulted positive for EBV latency and lytic markers, strongly indicating EBV persistence and reactivation as a common feature of MG patients' thymuses (**Figure 2**). In details, by ISH, variable number of EBERs-positive cells was detected in medullary infiltrates of all the MG thymuses analyzed, particularly in GCs of hyperplastic MG thymuses. Accordingly, IHC results showed expression of EBV latency proteins EBNA2, LMP1, and LMP2A in cells scattered throughout the thymic medulla and in GCs. The early BFRF1 and BMRF1, and the late p160 and gp350/220 lytic phase EBV proteins were also found in most MG thymuses, but not in control tissues, indicating EBV reactivation. Double immunofluorescence and confocal microscopy then revealed that latently infected cells were diffuse infiltrating and GC B-cells, whereas plasma blasts, mainly located around GCs, were positive for the lytic markers [85]. By PCR approaches, latent EBNA1 and LMP2A transcripts, and the early BZLF1 lytic transcript, along with EBV DNA, were detected in MG thymuses, but not in normal thymuses, thus confirming the active EBV infection. Due to the EBV properties to activate and immortalize B-cells, our findings thus suggested a critical contribution of the virus to intra-thymic B-cell dysfunction and B-cell-mediated autoimmunity in MG patients [85].

In a following study, we extended the analysis of EBV to additional MG thymuses (n = 19). Real-time PCR for EBV DNA (*Bam*HI-W repeat region), latent (EBER1, EBNA1, LMP1) and lytic (BZLF1) transcripts, and IHC for LMP1 and BZLF1 proteins, confirmed an active EBV infection in the thymus of MG patients

Figure 2.
Detection of EBV markers in follicular hyperplastic MG thymus (MG-FH), and MG thymoma (MG-T), but not in non-MG thymoma (No MG-T) and normal thymus. Representative images of immunohistochemistry and immunofluorescence stainings showing: EBER-positive cells and cells positive for latent LMP2A and lytic BMRF1 proteins in MG-FH; latent EBNA1-positive cells and CD20-positive B-cells expressing LMP1 in MG thymomas; absence of EBNA1-positive and LMP2A-positive cells in No MG-T; absence of LMP1-positive cells in normal thymuses.

but not in controls, thereby reinforcing the idea that the virus may significantly contribute to autoimmunity development or maintenance in MG thymus [86]. Similar results were found in MG thymuses from corticosteroid-naïve and -treated patients, indicating that the EBV infection profile we observed in our MG thymuses was not due to the immunosuppressive treatment [85, 86].

In contrast to our outcomes, Meyer and colleagues [94] and Kakalacheva and colleagues [95] reported absence or very low presence of EBV in MG thymuses. The first group performed ISH on formalin-fixed, paraffin-embedded MG thymic tissues (n = 44) and did not observe EBER-positive cells, likewise on cases of Hashimoto thyroiditis (n = 25), except for an isolate case in which rare EBER-positive lymphocytes were detected. Moreover, EBNA1 or BZLF1-positive cells were absent in MG thymuses or Hashimoto thyroiditis by IHC, whereas in infectious mononucleosis rare scattered positive cells were detected [94]. Kakalacheva *et al.* detected minimal levels of viral DNA in 6 of 16 hyperplastic MG thymuses, indicating rarity of viral copy numbers, confirmed by the observation of rare positivity for EBERs and viral proteins in the thymic sections by ISH and IHC [95]. Discrepancies between our data and data from these two studies may be due to sampling from patients with different clinical features, or to the use of different methods and tools with different sensitivity. In the article of Meyer et al., the cohort is apparently not homogeneous, and no description of treatment, nor clear indication of the patients' disease status, is given. Patients analyzed by Kakalacheva *et al.* did not require immunosuppression, so they maybe had a mild phenotype. As regards to the methods for EBV marker detection, tissue processing and procedures applied in these studies are different from ours, and perhaps less sensitive, or not optimized for detection of EBV in non-malignant

pathological conditions [96]. All these issues need to be solved, to confirm data on EBV in MG thymus, and a correct approach could be to find an agreement on the best procedures to analyze EBV infection in the thymus.

To better understand the EBV role in MG pathogenesis, we recently investigated the potential cross talk between TLRs and EBV in rising or sustaining self-reactivity. In details, since EBV molecules (EBERs, EBV DNA) are known to activate TLR7 and TLR9, and considering that these two receptors have super-addictive effects on EBV-induced B-cell activation and transformation process [66, 97], we analyzed their expression in EBV-positive MG and EBV-negative normal thymuses. We revealed an increased percentage of proliferating B-cells positive for EBV markers, and overexpressing TLR7 and TLR9, in EBV-positive hyperplastic MG thymuses compared to controls [63]. Our overall data thus indicated for the first time that aberrant EBV-driven TLR7 and TLR9 signaling in MG thymuses might contribute to abnormal B-cell activation and proliferation, in turn promoting or perpetuating B-cell-mediated autoimmunity in MG patients.

5.2 EBV in MG thymoma

Since the 1980s, the involvement of EBV in thymoma, associated or not with MG, has long been investigated. However, data obtained by the different studies were con-trasting: (i) McGuire et al. found EBV DNA in three thymomas, of which two were from MG patients [91]; (ii) absence of EBV in thymic epithelial tumors was reported by Inghirami *et al.* and Engel *et al.* [98, 99]; (iii) Chen et al. reported EBV DNA sig-nals in eight out of 21 thymic carcinoma with lymphoepithelioma-like morphology, a subtype not currently included in the WHO classification, but they did not specify whether EBV-positive tumors were from MG patients [100]; and (iv) Takeuchi et al. detected EBV-infected lymphocytes in one out of 11 thymomas not associated with MG [101]. Recently, an antiviral gene signature was identified in MG thymomas by Cufi and colleagues, consisting in a significant overexpression of type I IFNs and components of the TLR3 signaling pathways [102], thus opening the hypothesis of a viral etiology also for MG associated with thymoma. The same authors tried to identify the presence of potential pathogens in the MG thymomas focusing on human papillomavirus (HPV) and EBV, but no viral DNA was detected [102].

Recently, by combining ISH, IHC and molecular techniques, our group dem-onstrated the presence of latency EBV markers in MG-associated thymomas, but only rarely in thymomas from patients without MG [87]. Specifically, by real-time PCR we showed a significantly higher frequency of EBV DNA and EBER1 detec-tion in MG (53.8% and 84.6%) than non-MG (21.4%) thymomas, with the higher viral load and EBER1 levels being mainly observed in B2 and B2-mixed tumors, the WHO subtypes most frequently associated with MG. These data were in contrast with data on EBV DNA from Cufi and colleagues [102], likely because of the differ-ent sensitivity of the protocol used for the viral genome detection.

We then confirmed our molecular results by ISH, which showed the presence of cells positive for EBERs in MG, but not in non-MG thymomas [87]. Latent EBNA2 and late gp350/220 lytic transcripts were undetectable in all thymomas, except one, as well as the early lytic transcript BZLF1, thus revealing that early infection and EBV reactivation are very rare event in thymomas. By IHC analysis, we confirmed EBV persistence in MG thymomas, but not in thymomas without MG, and identified the phenotype of EBV-positive cells, that were B-cells positive for the EBV latency proteins EBNA1, LMP1, and LMP2, diffused throughout the MG neoplastic tissues (**Figure 2**). Based on the EBV gene expression pattern, we suggested EBV latency type II in MG thymomas. Due to the absence of EBV in thymic epithelial cells, these results thus revealed an association of EBV with B-cell-mediated autoimmunity in MG associated

with thymoma, rather than with thymic neoplastic transformation [87]. We also found higher TLR3 transcriptional levels in MG than non-MG thymomas. Transcriptional levels of this receptor positively correlate with EBER1 levels, supporting a possible role for EBER1 in inducing persistent TLR3 signaling pathways in thymoma from MG patients [87]. Due to the previously described role of TLR3 in triggering an anti-AChR autoimmune response [62], our findings strengthened the idea of a critical contribution of EBV to B-cell-mediated autoimmunity via TLR3 in these pathological tissues. Indeed, the activation of EBV-driven TLR3 signaling may well contribute to create an altered tumor microenvironment, which supports the recruitment of peripheral B-cells, and thus B-cell dysregulation and tolerance disruption.

6. Conclusions

EBV has been associated with several autoimmune diseases by sero-epidemiological and immunological data. Our findings revealed for the first time a contribution of EBV to the intra-thymic MG pathogenesis, thus allowing to include MG among the autoimmune conditions associated with the virus. Based on our results and literature data, we postulate that, in the context of a genetically susceptible background, EBV persistence and reactivation into the thymus may favor B-cell dysregulation, TLR over-stimulation, inflammation, and B-cell-mediated auto-immune response to AChR, which can be perpetuated in the periphery. Further advancement in the knowledge of the exact involvement of EBV and TLRs in MG pathogenesis could set the basis for novel investigations aimed at developing innovative therapeutic applications targeting EBV-positive cells, TLRs, or components of their signaling pathways to counteract autoimmunity in MG and potentially other EBV-associated autoimmune diseases.

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References

[1] Mantegazza R, Bernasconi P, Cavalcante P. Myasthenia gravis: From autoantibodies to therapy. Current Opinion in Neurology. 2018;**31**(5):517-525. DOI: 10.1097/WCO.0000000000000596

[2] Berrih-Aknin S. Myasthenia gravis: Paradox versus paradigm in autoimmunity. Journal of Autoimmunity. 2014;**52**:1-28. DOI: 10.1016/j.jaut.2014.05.001

[3] Berrih-Aknin S, Le Panse R. Myasthenia gravis: A comprehensive review of immune dysregulation and etiological mechanisms. Journal of Autoimmunity. 2014;**52**:90-100. DOI: 10.1016/j.jaut.2013.12.011

[4] Cavalcante P, Barzago C, Baggi F, et al. Toll-like receptors 7 and 9 in myasthenia gravis thymus: Amplifiers of autoimmunity? Annals of the New York Academy of Sciences. 2018;**1413**(1):11-24. DOI: 10.1111/nyas.13534

[5] Hehir MK, Silvestri NJ. Generalized myasthenia gravis: Classification, clinical presentation, natural history, and epidemiology. Neurologic Clinics. 2018;**36**:253-260. DOI: 10.1016/j.ncl.2018.01.002

[6] Wendell LC, Levine JM. Myasthenic crisis. Neurohospitalist. 2011;**1**:16-22. DOI: 10.1177/1941875210382918

[7] Vincent A, Huda S, Cao M, et al. Serological and experimental studies in different forms of myasthenia gravis. Annals of the New York Academy of Sciences. 2018;**1413**:143-153. DOI: 10.1111/nyas.13592

[8] Mantegazza R, Cavalcante P. Diagnosis and treatment of myasthenia gravis. Current Opinion in Rheumatology. 2019;**31**(6):623-633. DOI: 10.1097/BOR.0000000000000647

[9] Gilhus NE, Skeie GO, Romi F, et al. Myasthenia gravis—Autoantibody characteristics and their implications for therapy. Nature Reviews. Neurology. 2016;**12**(5):259-268. DOI: 10.1038/nrneurol.2016.44

[10] Leite MI, Jacob S, Viegas S, et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. Brain. 2008;**131**(Pt7):1940-1952. DOI: 10.1093/brain/awn092

[11] Tsonis AI, Zisimopoulou P, Lazaridis K, et al. MuSK autoantibodies in myasthenia gravis detected by cell based assay—A multinational study. Journal of Neuroimmunology. 2015;**284**:10-17. DOI: 10.1016/j.jneuroim.2015.04.015

[12] Zisimopoulou P, Evangelakou P, Tzartos J, et al. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. Journal of Autoimmunity. 2014;**52**:139-145. DOI: 10.1016/j.jaut.2013.12.004

[13] Cavalcante P, Cufi P, Mantegazza R, et al. Etiology of myasthenia gravis: Innate immunity signature in pathological thymus. Autoimmunity Reviews. 2013;**12**(9):863-874. DOI: 10.1016/j.autrev.2013.03.010

[14] Marx A, Porubsky S, Belharazem D, et al. Thymoma related myasthenia gravis in humans and potential animal models. Experimental Neurology. 2015;**270**:55-65. DOI: 10.1016/j.expneurol.2015.02.010

[15] Meriggioli MN, Sanders DB. Muscle autoantibodies in myasthenia gravis: Beyond diagnosis? Exp Rev Clin Immunol. 2012;**8**(5):427-438. DOI: 10.1586/eci.12.34

[16] Casetta I, Groppo E, De Gennaro R, et al. Myasthenia gravis: A changing pattern of incidence. Journal of

Neurology. 2010;**257**(12):2015-2019. DOI: 10.1007/s00415-010-5651-z

[17] Gilhus NE, Verschuuren JJ. Myasthenia gravis: Subgroup classification and therapeutic strategies. Lancet Neurology. 2015;**14**(10):1023-1036. DOI: 10.1016/S1474-4422(15)00145-3

[18] Evoli A, Alboini PE, Damato V, et al. Myasthenia gravis with antibodies to MuSK: An update. Annals of the New York Academy of Sciences. 2018;**1412**:82-89. DOI: 10.1111/nyas.13518

[19] Cordts I, Bodart N, Hartmann K, et al. Screening for lipoprotein receptor-related protein 4-, agrin-, and titin-antibodies and exploring the autoimmune spectrum in myasthenia gravis. Journal of Neurology. 2017;**264**(6):1193-1203. DOI: 10.1007/s00415-017-8514-z

[20] Conti-Fine BM, Milani M, Kaminski HJ. Myasthenia gravis: Past, present, and future. The Journal of Clinical Investigation. 2006;**116**(11):2843-2854. DOI: 10.1172/JCI29894

[21] Koneczny I, Stevens JA, De Rosa A, et al. IgG4 autoantibodies against muscle-specific kinase undergo fab-arm exchange in myasthenia gravis patients. Journal of Autoimmunity. 2017;**77**:104-115. DOI: 10.1016/j.jaut.2016.11.005

[22] Huijbers MG, Zhang W, Klooster R, et al. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**(51):20783-20788. DOI: 10.1073/pnas.1313944110

[23] Kawakami Y, Ito M, Hirayama M, et al. Anti-MuSK autoantibodies block binding of collagen Q to MuSK. Neurology. 2011;**77**(20):1819-1826. DOI: 10.1212/WNL.0b013e318237f660

[24] Viegas S, Jacobson L, Waters P, et al. Passive and active immunization models of MuSK-Ab positive myasthenia: Electrophysiological evidence for pre and postsynaptic defects. Experimental Neurology. 2012;**234**(2):506-512. DOI: 10.1016/j.expneurol.2012.01.025

[25] Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunology Today. 1993;**14**(9):426-430. DOI: 10.1016/0167-5699(93)90244-F

[26] Mori S, Motohashi N, Takashima R, et al. Immunization of mice with LRP4 induces myasthenia similar to MuSK-associated myasthenia gravis. Experimental Neurology. 2017;**297**:158-167. DOI: 10.1016/j.expneurol.2017.08.006

[27] Shen C, Lu Y, Zhang B, et al. Antibodies against low-density lipoprotein receptor-related protein 4 induce myasthenia gravis. The Journal of Clinical Investigation. 2013;**123**(12):5190-5202. DOI: 10.1172/JCI66039

[28] Kurd N, Robey EA. T-cell selection in the thymus: A spatial and temporal perspective. Immunological Reviews. 2016;**271**(1):114-126. DOI: 10.1111/imr.12398

[29] Wolfe GI, Kaminski HJ, Aban IB, et al. Randomized trial of thymectomy in myasthenia gravis. The New England Journal of Medicine. 2016;**375**:511-522. DOI: 10.1056/NEJMoa1602489

[30] Cavalcante P, Le Panse R, Berrih-Aknin S, et al. The thymus in myasthenia gravis: Site of "innate autoimmunity"? Muscle & Nerve. 2011;**44**(4):467-484. DOI: 10.1002/mus.22103

[31] Domeier PP, Schell SL, Rahman ZS. Spontaneous germinal centers and autoimmunity. Autoimmunity. 2017;**50**(1):4-18. DOI: 10.1080/08916934.2017.1280671

[32] Pitzalis C, Jones GW, Bombardieri M, et al. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. Nature Reviews. Immunology. 2014;**14**(7):447-462. DOI: 10.1038/nri3700

[33] Aloisi F, Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. Nature Reviews. Immunology. 2006;**6**(3):205-217. DOI: 10.1038/nri1786

[34] Berrih-Aknin S, Ruhlmann N, Bismuth J, et al. CCL21 overexpressed on lymphatic vessels drives thymic hyperplasia in myasthenia. Annals of Neurology. 2009;**66**(4):521-531. DOI: 10.1002/ana.21628

[35] Roxanis I, Micklem K, McConville J, et al. Thymic myoid cells and germinal center formation in myasthenia gravis; possible roles in pathogenesis. Journal of Neuroimmunology. 2002;**125**(1-2):185-197. DOI: 10.1016/s0165-5728(02)00038-3

[36] Schönbeck S, Padberg F, Hohlfeld R, Wekerle H. Transplantation of thymic autoimmune microenvironment to severe combined immunodeficiency mice. A new model of myasthenia gravis. The Journal of Clinical Investigation. 1992;**90**(1):245-250. DOI: 10.1172/JCI115843

[37] Wakkach A, Guyon T, Bruand C, et al. Expression of acetylcholine receptor genes in human thymic epithelial cells: Implications for myasthenia gravis. Journal of Immunology. 1996;**157**(8): 3752-3760

[38] Vincent A, Willcox N, Hill M, et al. Determinant spreading and immune responses to acetylcholine receptors in myasthenia gravis. Immunological Reviews. 1998;**164**:157-168. DOI: 10.1111/j.1600-065x.1998.tb01217.x

[39] Villadangos JA, Heath WR, Carbone FR. Outside looking in: The inner workings of the cross-presentation pathway within dendritic cells. Trends in Immunology. 2007;**28**(2):45-47. DOI: 10.1016/j.it.2006.12.008

[40] Bornemann A, Kirchner T. Thymic myoid cell turnover in myasthenia gravis patients and in normal controls. Virchows Archiv. 1998;**432**(4):357-361. DOI: 10.1007/s004280050178

[41] Leite MI, Jones M, Ströbel P, et al. Myasthenia gravis thymus: Complement vulnerability of epithelial and myoid cells, complement attack on them, and correlations with autoantibody status. The American Journal of Pathology. 2007 Sep;**171**(3):893-905. DOI: 10.2353/ajpath.2007.070240

[42] Poëa-Guyon S, Christadoss P, Le Panse R, et al. Effects of cytokines on acetylcholine receptor expression: Implications for myasthenia gravis. Journal of Immunology. 2005;**174**(10):5941-5949. DOI: 10.4049/jimmunol.174.10.5941

[43] Cron MA, Maillard S, Villegas J, et al. Thymus involvement in early-onset myasthenia gravis. Annals of the New York Academy of Sciences. 2018;**1412**(1):137-145. DOI: 10.1111/nyas.13519

[44] Cufi P, Dragin N, Ruhlmann N, et al. Central role of interferon-beta in thymic events leading to myasthenia gravis. Journal of Autoimmunity. 2014;**52**:44-52. DOI: 10.1016/j.jaut.2013.12.016

[45] Gradolatto A, Nazzal D, Truffault F, et al. Both Treg cells and T conv cells are defective in the myasthenia gravis thymus: Roles of IL-17 and TNF-α. Journal of Autoimmunity. 2014;**52**:53-63. DOI: 10.1016/j.jaut.2013.12.015

[46] Bernard C, Frih H, Pasquet F, et al. Thymoma associated with autoimmune diseases: 85 cases and literature review. Autoimmunity Reviews. 2016;**15**(1):82-92. DOI: 10.1016/j.autrev.2015.09.005

[47] Marx A, Chan JK, Coindre JM, et al. The 2015 World Health Organization classification of tumors of the thymus: Continuity and changes. Journal of Thoracic Oncology. 2015;**10**(10):1383-1395. DOI: 10.1097/JTO.0000000000000654

[48] Maggi L, Andreetta F, Antozzi C, et al. Thymoma-associated myasthenia gravis: outcome, clinical and pathological correlations in 197 patients on a 20-year experience. J Neuroimmunol. 2008;**201-202**:237-244. DOI: 10.1016/j.jneuroim.2008.07.012

[49] Marx A, Willcox N, Leite MI, et al. Thymoma and paraneoplastic myasthenia gravis. Autoimmunity. 2010;**43**(5-6):413-427. DOI: 10.3109/08916930903555935

[50] Savino W, Manganella G, Verley JM, et al. Thymoma epithelial cells secrete thymic hormone but do not express class II antigens of the major histocompatibility complex. The Journal of Clinical Investigation. 1985;**76**(3):1140-1146. DOI: 10.1172/JCI112069

[51] Lefeuvre CM, Payet CA, Fayet OM, et al. Risk factors associated with myasthenia gravis in thymoma patients: The potential role of thymic germinal centers. Journal of Autoimmunity. 2020;**106**:102337. DOI: 10.1016/j.jaut.2019.102337

[52] Wolfe GI, Kaminski HJ, Aban IB, et al. MGTX study group. Long-term effect of thymectomy plus prednisone versus prednisone alone in patients with non-thymomatous myasthenia gravis: 2-year extension of the MGTX randomised trial. Lancet Neurology. 2019;**18**:259-268. DOI: 10.1016/S1474-4422(18)30392-2

[53] Okumura M, Ohta M, Takeuchi Y, et al. The immunologic role of thymectomy in the treatment of myasthenia gravis: Implication of thymus-associated B-lymphocyte subset

in reduction of the anti-acetylcholine receptor antibody titer. The Journal of Thoracic and Cardiovascular Surgery. 2003;**126**(6):1922-1928. DOI: 10.1016/s0022-5223(03)00938-3

[54] Fujii Y, Monden Y, Hashimoto J, Nakahara K, Kawashima Y. Acetylcholine receptor antibody-producing cells in thymus and lymph nodes in myasthenia gravis. Clinical Immunology and Immunopathology. 1985;**34**(1):141-146. DOI: 10.1016/0090-1229(85)90018-2

[55] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. Nature Immunology. 2010;**11**(5):373-384. DOI: 10.1038/ni.1863

[56] Mohammad Hosseini A, Majidi J, Baradaran B, Yousefi M. Toll-like receptors in the pathogenesis of autoimmune diseases. Adv Pharm Bull. 2015;**5**:605-614. DOI: 10.15171/apb.2015.082

[57] Duffy L, O'Reilly SC. Toll-like receptors in the pathogenesis of autoimmune diseases: Recent and emerging translational developments. Immunotargets Ther. 2016;**5**:69-80. Published 2016 Aug 22. DOI: 10.2147/ITT.S89795

[58] Soni C, Wong EB, Domeier PP, et al. B cell-intrinsic TLR7 signaling is essential for the development of spontaneous germinal centers. Journal of Immunology. 2014;**193**(9):4400-4414. DOI: 10.4049/jimmunol.140172

[59] Bernasconi P, Barberis M, Baggi F, et al. Increased toll-like receptor 4 expression in thymus of myasthenic patients with thymitis and thymic involution. The American Journal of Pathology. 2005;**167**(1):129-139. DOI: 10.1016/S0002-9440(10)62960-4

[60] Cordiglieri C, Marolda R, Franzi S, et al. Innate immunity in myasthenia

gravis thymus: Pathogenic effects of toll-like receptor 4 signaling on autoimmunity. Journal of Autoimmunity. 2014;**52**:74-89. DOI: 10.1016/j.jaut.2013.12.013

[61] Cufi P, Dragin N, Weiss JM, et al. Implication of double-stranded RNA signaling in the etiology of autoimmune myasthenia gravis. Annals of Neurology. 2013;**73**(2):281-293. DOI: 10.1002/ana.23791

[62] Robinet M, Maillard S, Cron MA, et al. Review on toll-like receptor activation in myasthenia gravis: Application to the development of new experimental models. Clinical Reviews in Allergy and Immunology. 2017;**52**(1):133-147. DOI: 10.1007/s12016-016-8549-4

[63] Cavalcante P, Galbardi B, Franzi S, et al. Increased expression of toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein-Barr virus infection. Immunobiology. 2016;**221**(4):516-527. DOI: 10.1016/j.imbio.2015.12.007

[64] Hennessy EJ, Parker AE, O'Neill LA. Targeting toll-like receptors: Emerging therapeutics? Nature Reviews. Drug Discovery. 2010;**9**(4):293-307. DOI: 10.1038/nrd3203

[65] Thorley-Lawson DA. Epstein-Barr virus: Exploiting the immune system. Nature Reviews. Immunology. 2001;**1**(1):75-82. DOI: 10.1038/35095584

[66] Ning S. Innate immune modulation in EBV infection. Herpesviridae. 2011;**2**(1):1. DOI: 10.1186/2042-4280-2-1

[67] Tattevin P, Le Tulzo Y, Minjolle S, et al. Increasing incidence of severe Epstein-Barr virus-related infectious mononucleosis: Surveillance study. Journal of Clinical Microbiology. 2006;**44**(5):1873-1874. DOI: 10.1128/JCM.44.5.1873-1874.2006

[68] Lassmann H, Niedobitek G, Aloisi F, Middeldorp JM, NeuroproMiSe EBV Working Group. Epstein-Barr virus in the multiple sclerosis brain: A controversial issue-report on a focused workshop held in the Centre for Brain Research of the Medical University of Vienna, Austria. Brain. 2011;**134**: 2772-2786. DOI: 10.1093/brain/awr197

[69] Kalla M, Hammerschmidt W. Human B cells on their route to latent infection-early but transient expression of lytic genes of Epstein-Barr virus. European Journal of Cell Biology. 2012;**91**(1):65-69. DOI: 10.1016/j.ejcb.2011.01.014

[70] Niller HH, Wolf H, Minarovits J. Regulation and dysregulation of Epstein-Barr virus latency: Implications for the development of autoimmune diseases. Autoimmunity. 2008;**41**(4):298-328. DOI: 10.1080/08916930802024772

[71] Henderson S, Huen D, Rowe M, Dawson C, Johnson G, Rickinson A. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proceedings of the National Academy of Sciences of the United States of America. 1993;**90**(18):8479-8483. DOI: 10.1073/pnas.90.18.8479

[72] Iizasa H, Kim H, Kartika AV, Kanehiro Y, Yoshiyama H. Role of viral and host microRNAs in immune regulation of Epstein-Barr virus-associated diseases. Frontiers in Immunology. 2020;**11**:367. DOI: 10.3389/fimmu.2020.00367

[73] Taylor GS, Long HM, Brooks JM, et al. The immunology of Epstein-Barr virus-induced disease. Annual Review of Immunology. 2015;**33**:787-821. DOI: 10.1146/annurev-immunol-032414-112326

[74] Serafini B, Rosicarelli B, Franciotta D, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain.

The Journal of Experimental Medicine. 2007;**204**(12):2899-2912. DOI: 10.1084/jem.20071030

[75] Croia C, Astorri E, Murray-Brown W, et al. Implication of Epstein-Barr virus infection in disease-specific autoreactive B cell activation in ectopic lymphoid structures of Sjögren's syndrome. Arthritis & Rhematology. 2014;**66**(9):2545-2557. DOI: 10.1002/art.38726

[76] Croia C, Serafini B, Bombardieri M, et al. Epstein-Barr virus persistence and infection of autoreactive plasma cells in synovial lymphoid structures in rheumatoid arthritis. Annals of the Rheumatic Diseases. 2013;**72**(9):1559-1568. DOI: 10.1136/annrheumdis-2012-202352

[77] Münz C, Lünemann JD, Getts MT, et al. Antiviral immune responses: Triggers of or triggered by autoimmunity? Nature Reviews. Immunology. 2009;**9**(4):246-258. DOI: 10.1038/nri2527

[78] Dreyfus DH. Genetics and molecular biology of Epstein-Barr virus-encoded BART MicroRNA: A paradigm for viral modulation of host immune response genes and genome stability. Journal of Immunology Research. 2017;**2017**:4758539. DOI: 10.1155/2017/4758539

[79] Lünemann JD, Edwards N, Muraro PA, et al. Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. Brain. 2006;**129**:1493-1506. DOI: 10.1093/brain/awl067

[80] Lünemann JD, Jelcić I, Roberts S, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. The Journal of Experimental Medicine. 2008;**205**(8):1763-1773. DOI: 10.1084/jem.20072397

[81] Poole BD, Gross T, Maier S, Harley JB, James JA. Lupus-like autoantibody development in rabbits and mice after immunization with EBNA-1 fragments. Journal of Autoimmunity. 2008;**31**(4):362-371. DOI: 10.1016/j.jaut.2008.08.007

[82] Balandraud N, Roudier J. Epstein-Barr virus and rheumatoid arthritis. Joint, Bone, Spine. 2018;**85**(2):165-170. DOI: 10.1016/j.jbspin.2017.04.011

[83] Tzartos JS, Khan G, Vossenkamper A, et al. Association of innate immune activation with latent Epstein-Barr virus in active MS lesions. Neurology. 2012;**78**(1):15-23. DOI: 10.1212/WNL.0b013e31823ed057

[84] Severa M, Giacomini E, Gafa V, et al. EBV stimulates TLR- and autophagy-dependent pathways and impairs maturation in plasmacytoid dendritic cells: Implications for viral immune escape. European Journal of Immunology. 2013;**43**(1):147-158. DOI: 10.1002/eji.201242552

[85] Cavalcante P, Serafini B, Rosicarelli B, et al. Epstein-Barr virus persistence and reactivation in myasthenia gravis thymus. Annals of Neurology. 2010;**67**(6):726-738. DOI: 10.1002/ana.21902

[86] Cavalcante P, Maggi L, Colleoni L, et al. Inflammation and Epstein-Barr virus infection are common features of myasthenia gravis thymus: Possible roles in pathogenesis. Autoimmune Dis. 2011;**2011**:213092. DOI: 10.4061/2011/213092

[87] Cavalcante P, Marcuzzo S, Franzi S, et al. Epstein-Barr virus in tumor-infiltrating B cells of myasthenia gravis thymoma: An innocent bystander or an autoimmunity mediator? Oncotarget. 2017;**8**(56):95432-95449. DOI: 10.18632/oncotarget.20731

[88] Klavinskis LS, Willcox N, Oxford JS, et al. Antivirus antibodies in myasthenia

gravis. Neurology. 1985;**35**(9):1381-1384. DOI: 10.1212/wnl.35.9.1381

[89] Bhibhatbhan A, Kline G, Vincent A, et al. Anti-MuSK myasthenia gravis presenting with Epstein-Barr virus-associated mononucleosis and immune-mediated diabetes mellitus. Muscle & Nerve. 2007;**36**(2):264-266. DOI: 10.1002/mus.20746

[90] Csuka D, Banati M, Rozsa C, et al. High anti-EBNA-1 IgG levels are associated with early-onset myasthenia gravis. European Journal of Neurology. 2012;**19**(6):842-846. DOI: 10.1111/j.1468-1331.2011.03636.x

[91] McGuire LJ, Huang DP, Teoh R, et al. Epstein-Barr virus genome in thymoma and thymic lymphoid hyperplasia. The American Journal of Pathology. 1988;**131**(3):385-390

[92] Aoki T, Drachman DB, Asher DM, et al. Attempts to implicate viruses in myasthenia gravis. Neurology. 1985;**35**(2):185-192. DOI: 10.1212/wnl.35.2.185

[93] Klavinskis LS, Willcox HN, Richmond JE, et al. Attempted isolation of viruses from myasthenia gravis thymus. Journal of Neuroimmunology. 1986;**11**(4):287-299. DOI: 10.1016/0165-5728(86)90082-2

[94] Meyer M, Höls AK, Liersch B, et al. Lack of evidence for Epstein-Barr virus infection in myasthenia gravis thymus. Annals of Neurology. 2011;**70**(3):515-518. DOI: 10.1002/ana.22522

[95] Kakalacheva K, Maurer MA, Tackenberg B, et al. Intrathymic Epstein-Barr virus infection is not a prominent feature of myasthenia gravis. Annals of Neurology. 2011;**70**(3):508-514. DOI: 10.1002/ana.22488

[96] Serafini B, Cavalcante P, Bernasconi P, et al. Epstein-Barr virus in myasthenia gravis thymus: A matter of debate. Annals of Neurology. 2011;**70**(3):519. DOI: 10.1002/ana.22544

[97] Iskra S, Kalla M, Delecluse HJ, et al. Toll-like receptor agonists synergistically increase proliferation and activation of B cells by Epstein-Barr virus. Journal of Virology. 2010;**84**(7):3612-3623. DOI: 10.1128/JVI.01400-09

[98] Inghirami G, Chilosi M, Knowles DM. Western thymomas lack Epstein-Barr virus by southern blotting analysis and by polymerase chain reaction. American journal of pathology. 1990;**136**:1429-1436

[99] Engel PJ. Absence of latent Epstein-Barr virus in thymic epithelial tumors as demonstrated by Epstein-Barr-encoded RNA(EBER) in situ hybridization. APMIS. 2000;**108**:393-397. DOI: 10.1034/j.1600-0463.2000.d01-74.x

[100] Chen PC, Pan CC, Yang AH, et al. Detection of Epstein-Barr virus genome within thymic epithelial tumours in Taiwanese patients by nested PCR, PCR in situ hybridization, and RNA in situ hybridization. The Journal of Pathology. 2002;**197**(5):684-688. DOI: 10.1002/path.1141

[101] Takeuchi H, Fujita H, Iwasaki F, et al. A case of Epstein- Barr virus (EBV)-associated thymic carcinoid and investigation of existence of EBV-infected cells in thymus and thymic tumors. Journal of Clinical Microbiology. 2004;**42**(6):2850-2854. DOI: 10.1128/JCM.42.6.2850-2854.2004

[102] Cufi P, Soussan P, Truffault F, et al. Thymoma-associated myasthenia gravis: On the search for a pathogen signature. Journal of Autoimmunity. 2014;**52**:29-35. DOI: 10.1016/j.jaut.2013.12.018