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# Increased expression of Toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein–Barr virus infection

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ABSTRACT

Considerable data implicate the thymus as the main site of autosensitization to the acetylcholine receptor in myasthenia gravis (MG), a B-cell-mediated autoimmune disease affecting the neuromuscular junction. We recently demonstrated an active Epstein–Barr virus (EBV) infection in the thymus of MG patients, suggesting that EBV might contribute to the onset or maintenance of the autoimmune response within MG thymus, because of its ability to activate and immortalize autoreactive B cells. EBV has been reported to elicit and modulate Toll-like receptor (TLR) 7- and TLR9-mediated innate immune responses, which are known to favor B-cell dysfunction and autoimmunity. Aim of this study was to investigate whether EBV infection is associated with altered expression of TLR7 and TLR9 in MG thymus. By real-time PCR, we found that TLR7 and TLR9 mRNA levels were significantly higher in EBV-positive MG compared to EBV-negative normal thymuses. By confocal microscopy, high expression levels of TLR7 and TLR9 proteins were observed in B cells and plasma cells of MG thymic germinal centers (GCs) and lymphoid infiltrates, where the two receptors co-localized with EBV antigens. An increased frequency of Ki67-positive proliferating B cells was found in MG thymuses, where we also detected proliferating cells expressing TLR7, TLR9 and EBV antigens, thus supporting the idea that EBV-associated TLR7/9 signaling may promote abnormal B-cell activation and proliferation. Along with B cells and plasma cells, thymic epithelium, plasmacytoid dendritic cells and macrophages exhibited enhanced TLR7 and TLR9 expression in MG thymus; TLR7 was also increased in thymic myeloid dendritic cells and its transcriptional levels positively correlated with those of interferon (IFN)-β. We suggested that TLR7/9 signaling may be involved in antiviral type I IFN production and long-term inflammation in EBV-infected MG thymuses. Our overall findings indicate that EBV-driven TLR7- and TLR9-mediated innate immune responses may participate in the intra-thymic pathogenesis of MG.



*Abbreviations:* MG, myasthenia gravis; EBV, Epstein–Barr virus; TLR, Toll-like receptor; GC, germinal center; IFN, interferon; AChR, acetylcholine receptor; EBER, EBV-encoded small RNA; LCM, laser-capture microdissection; IRF8, interferon regulatory factor 8; LMP1, Epstein–Barr virus latent membrane protein 1; DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; TEC, thymic epithelial cells; pDC, plasmacytoid dendritic cell; mDC, myeloid dendritic cell; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; RIG-I, retinoic acid inducible gene-I.

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## 1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by fluctuating muscle weakness and fatigability, resulting from the production of autoantibodies against neuromuscular junction (NMJ) components. In most patients (>80%) the target of the autoimmune reaction is the postsynaptic acetylcholine receptor (AChR); less frequently, targets of autoimmunity are the muscle specific kinase receptor or the low-density lipoprotein receptor-related protein 4 (Berrih-Aknin and Le Panse, 2014).

Several lines of evidence support the involvement of the thymus in the pathogenesis of AChR-MG (Berrih-Aknin and Le Panse, 2014). In most AChR-positive MG patients thymus exhibits pathological changes, including hyperplasia, which is the most common alteration in early-onset (MG onset < 50 years of age) patients, and thymoma, which occurs most frequently in late-onset patients (>50 years) (Cavalcante et al., 2011a, 2013). Thymic hyperplasia is characterized by expanded perivascular spaces containing B-cell infiltrates, that can be organized into germinal centers (GCs) forming follicles (follicular hyperplasia) or distributed throughout the thymic medulla (diffuse hyperplasia or thymitis) (Cavalcante et al., 2011a; Müller-Hermelink et al., 1996; Berrih-Aknin et al., 2013). Autoreactive T cells and activated B cells producing autoantibodies can be isolated from hyperplastic MG thymus, indicating that the anti-AChR autoimmune reaction develops, and probably arises, within this organ (Sommer et al., 1990; Leprince et al., 1990; Hill et al., 2008).

Pathogen infections are suspected to play a role in autoimmune diseases through the induction of dysregulated Toll-like receptor (TLR)-mediated innate immune responses, which can lead to inflammation, general activation of the adaptive immune system and autosensitization (Hurst and von Landenberg, 2008; Münz et al., 2009). Growing evidence of chronic inflammation and TLR3 and 4 activation in MG thymus strongly supports the hypothesis that, in the context of a genetic susceptible background, persistent or dysregulated innate immune responses to an unknown "danger signal", such as a pathogen infection, might contribute to the intra-thymic MG etiology (Bernasconi et al., 2005; Cizeron-Clairac et al., 2008; Cufi et al., 2013; Cordiglieri et al., 2014). Among pathogens, Epstein–Barr virus (EBV), a human γ herpesvirus that infects most (90–95%) of the world population, is one of the main candidates suspected to play a role in initiation or exacerbation of autoimmune diseases, due to its ability to promote abnormal activation and survival of B cells, and to disrupt critical B-cell tolerance checkpoints (Swanson-Mungerson and Longnecker, 2007; Niller et al., 2011). Recently, we showed an active EBV infection in hyperplastic (both follicular and diffuse) and involuted MG thymuses, but not in normal control thymuses (Cavalcante et al., 2010, 2011b), suggesting that EBV could contribute to onset or perpetuation of autoimmunity within the thymus of MG patients. Experimental evidence showed that latent EBV proteins can interfere with normal B-cell functions through mechanisms which include an increased B-cell sensitivity to TLR stimulation (Wang et al., 2006). Indeed, along with antigen binding to the B-cell receptor and CD40 stimulation, TLR stimuli mediated by TLR7 and TLR9 provide additional co-stimulatory signals for proliferation and maturation of B cells, including autoreactive B cells (Crampton et al., 2010; Ruprecht and Lanzavecchia, 2006). TLR7 and TLR9 are intracellular endosomal-lysosomal receptors able to recognize viral single-stranded RNA (ssRNA) and unmethylated 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) bacterial or viral DNA, respectively (Kawai and Akira, 2010). In vitro and in vivo studies suggested that loss of TLR7 and TLR9 signaling regulation can lead to autoimmunity, due to the ability of these receptors to stimulate B-cell activation and autoantibody production (Marshak-Rothstein, 2006). A crosstalk between EBV, TLR7 and TLR9 can be hypothesized: (i) EBV can alter the expression of the two receptors in B cells (Martin et al., 2007); (ii) EBV itself can elicit TLR7- and TLR9-mediated signaling (Fiola et al., 2010; Ning, 2011); (iii) TLR7 and TLR9 signaling pathways have a "super-additive" effect on the EBV-driven B-cell activation and transformation process (Iskra et al., 2010).

In this study, we investigated the potential association of EBV infection with altered expression of TLR7 and TLR9 in MG thymuses. Our data demonstrate a significant contribution of the signaling pathways mediated by the two receptors to the intra-thymic pathogenesis of MG.

## 2. Material and methods

### 2.1. Thymus specimens

The study included 15 thymuses with follicular hyperplasia, 11 thymuses with diffuse hyperplasia and 10 involuted thymuses obtained from 29 female and 7 male MG patients (mean age at disease onset: 27.2 ± 9.6 years), who underwent thymectomy (mean age at thymic surgery: 29.6 ± 9.7 years). The study was approved by the Ethic Committee of the Neurological Institute 'Carlo Besta', and each patient provided written informed consent for thymectomy and use of thymus for research purposes.

Histological classification of thymuses was performed at the Department of Pathological Anatomy, Azienda Ospedaliera Bolognini (Seriate, Bergamo). Patients' clinical characteristics are summarized in Table 1. All MG thymuses included in this study resulted positive for intra-thymic EBV infection in our previous studies (Cavalcante et al., 2010, 2011b). As controls, we examined 10 non-pathological thymuses from patients undergoing cardiovascular surgery (4 females and 6 males; mean age at surgery: 24.6 ± 13.9); all were tested for the presence of EBV DNA and EBV-encoded small RNA 1 (EBER1), as previously described (Cavalcante et al., 2010, 2011b), and resulted EBV-negative.

For each thymus, some fragments were fixed in 10% formalin for histopathological classification; other fragments were snap-frozen and stored at −80 °C for immunohistochemistry and molecular analyses.

### 2.2. Laser-capture microdissection (LCM)

Six snap-frozen MG hyperplastic thymuses were subjected to LCM of GCs using a Nikon Eclipse TE2000-S microscope (Nikon GMBH, Germany), equipped with a laser microdissector CellCut (MMI). For each thymus, six to ten 15-μm thick serial sections were mounted on membrane slides for LCM, stained by 50% hematoxylin and fixed in RNase-free 75–100% ethanol. Sections before and after these series were stained for CD20, a B-cell marker, to identify GCs, as described below. From each MG thymic sample, at least 20 GCs (from consecutive serial sections) were microdissected and pooled in a single cap; sections devoid of microdissected GCs were collected in separate caps. Whole sections from 5 control thymuses were collected as controls. The isolated tissue fragments of each series were incubated in lysis buffer (RNeasy Micro kit, Qiagen, Valencia, CA) at 37 °C for 1 h and centrifuged at 800 × *g* for 5 min; lysates were then stored at −80 °C until use.

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