

# Review on Toll-Like Receptor Activation in Myasthenia Gravis: Application to the Development of New Experimental Models

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**Abstract** Abnormal toll-like receptor (TLR) activation and uncontrolled resolution of inflammation are suspected to play a key role in the development of autoimmune diseases. Acquired myasthenia gravis (MG) is an invalidating neuromuscular disease leading to muscle weaknesses. MG is mainly mediated by anti-acetylcholine receptor (AChR) autoantibodies, and thymic hyperplasia characterized by ectopic germinal centers is a common feature in MG. An abnormal expression of certain TLRs is observed in the thymus of MG patients associated with the overexpression of interferon (IFN)- $\beta$ , the orchestrator of thymic changes in MG. Experimental models have been developed for numerous autoimmune diseases. These models are induced by animal immunization with a purified antigen solubilized in complete Freund's adjuvant (CFA) containing heat-inactivated mycobacterium tuberculosis (MTB). Sensitization against the antigen is mainly due to the activation of TLR signaling pathways by the pathogen motifs displayed by MTB, and attempts have been made to substitute the use of CFA by TLR agonists. AChR emulsified in CFA is used to induce the classical experimental autoimmune MG model (EAMG). However, the TLR4 activator lipopolysaccharide (LPS) has proved to be efficient to replace MTB and induce a sensitization against

purified AChR. Poly(I:C), the well-known TLR3 agonist, is also able by itself to induce MG symptoms in mice associated with early thymic changes as observed in human MG. In this review, we discuss the abnormal expression of TLRs in MG patients and we describe the use of TLR agonists to induce EAMG in comparison with other autoimmune experimental models.

**Keywords** Autoimmune diseases · Inflammation · Innate immunity · Mouse experimental models · Complete Freund's adjuvant · Interferon type-I

## Abbreviations

AChR	Acetylcholine receptor
CFA	Complete Freund's adjuvant
EAMG	Experimental autoimmune myasthenia gravis
EBV	Epstein-Barr virus
EOMG	Early-onset MG
GC	Germinal center
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
IRF	IFN-regulatory factor
KO	Knock-out
MG	Myasthenia gravis
MyD88	Myeloid differentiation primary response gene 88
PBMCs	Peripheral blood mononuclear cells
Poly(I:C)	Polyinosinic-polycytidylic acid
TEC	Thymic epithelial cell
TLR	Toll-like receptor
TRIF	TIR domain-containing adapter-inducing interferon- $\beta$

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## Role of Thymic Inflammation in Myasthenia Gravis Development

### Introduction on Myasthenia Gravis

Autoimmune myasthenia gravis (MG) is a neuromuscular disorder characterized by a defective transmission of nerve impulses to muscles. This defect is caused by an autoimmune attack against components of the neuromuscular junction on the postsynaptic membrane of striated skeletal muscles. Around 85 to 90 % of MG patients have autoantibodies against the acetylcholine receptor (AChR) [1]. The other smaller groups of MG patients display autoantibodies against the muscle-specific kinase (MuSK) [2] or the low-density lipoprotein-related protein 4 (LRP4) [3] and even against agrin [4].

The characteristic feature of all MG forms is a fluctuating fatigable muscle weakness, which can range from a mild form affecting only the eye muscles to a severe form involving respiratory muscles [5]. Patients with anti-AChR antibodies can be divided into subgroups according to the age of onset. In MG patients, histological abnormalities are very often found in the thymus, which displays either thymic hyperplasia of lympho-proliferative origin in early-onset MG (EOMG) or thymoma in late-onset MG [6, 7]. In contrast, the thymus of MuSK-MG patients shows no pathological changes and beneficial effects of thymectomy have not been proven for this subgroup [8, 9]. There is still no clear information on the involvement of the thymus in the LRP4-MG patients.

### Thymic Abnormalities Observed in MG Patients

The thymus provides an essential and complex environment for the generation of the T cell repertoire. The differentiation of thymocytes occurs through interactions with stromal cells, while they are progressing in the different thymic compartments [10, 11], thymic epithelial cells (TECs) being the main thymic stromal cells. In thymic cortical regions, TECs mainly mediate the positive selection of thymocytes, while in medullary regions, TECs are involved in the negative selection of thymocytes. Medullary TECs express a wide range of tissue-specific antigens (TSAs) and mediate the depletion of autoreactive T cells [12]. Only a few thymocytes successfully pass the thymic selection and are exported to the periphery as CD4 or CD8 T cells where they will differentiate into different subsets.

In EOMG, the thymus is the site of profound structural alterations [6]. The main characteristics of MG thymuses are the development of ectopic GCs in perivascular spaces in medullary regions. However, active neoangiogenic processes are also observed in the thymus with the development of high endothelial venules (HEVs) around GCs, and of lymphatic vessels [13, 14, 1, 15]. The thymus in EOMG displays all the characteristics of tertiary lymphoid organs [16, 17]. The hyperplastic thymus includes all the components of the anti-

AChR response, AChR [18], B cells producing anti-AChR antibodies [19, 20], and anti-AChR autoreactive T cells [21]. Thymic hyperplasia is especially correlated with the anti-AChR antibody titers. All these observations support the role of the thymus in the pathogenesis of MG, and thymectomy is often advised for AChR-MG patients, resulting in a decrease in anti-AChR antibodies [22] and a slow but effective improvement of symptoms over a few years [23, 9].

Thymic changes in MG are driven by the abnormal overexpression of chemokines such as CXCL13, CCL21, CCL19, CXCL12, CXCL10, CCL17, and CCL22 [24, 14, 25]. The implication of CXCL13 and CCL21 in thymic hyperplasia has been particularly studied as these two chemokines are involved in B-cell recruitment and GC development [26, 27].

CXCL13 is known to be produced by GCs in secondary lymphoid organs but in EOMG, CXCL13 is overexpressed by medullary TECs [28, 29, 30] and several indications suggest that CXCL13 could be involved in the recruitment of B cells: (1) thymic extracts from MG patients have a strong chemoattractive effect on B cells, (2) this effect is reduced when using anti-CXCL13 blocking antibodies, and (3) the thymus of MG patients under corticotherapy shows a normalized level of CXCL13 together with a reduced number of GCs [28]. Moreover, thymic and serum levels of CXCL13 were shown to correlate with disease severity [28, 30, 31].

The active neoangiogenesis processes are also crucial in the recruitment of peripheral cells via interactions with chemokines. In EOMG patients, thymic HEVs selectively expressed CXCL12, and antigen-presenting cells such as macrophages, DCs, and B cells expressing CXCR4 (the CXCL12 receptor) are detected inside and around thymic HEVs [15]. Thymic hyperplasia in MG patients is also specifically associated with the thymic overexpression of CCL21 and CCL19, two chemokines interacting with the CCR7 receptor. CCL21 overexpression in hyperplastic MG thymuses is due to lymphatic endothelial vessels that could also lead to a recruitment of peripheral cells, such as sensitized DCs but also T and B cells [13].

### Central Role of Type-I Interferons in Thymic Changes

The role of inflammatory cytokines is crucial for the autoimmune sensitization against AChR in the thymus of MG patients. Type-I interferons (IFN-I) are pleiotropic cytokines involved in innate immune responses and the regulation of the pro-inflammatory responses. The implication of IFN-I in MG has long been suggested in various ways: (1) clinical reports demonstrate the development of MG after IFN- $\alpha$ - or IFN- $\beta$ -based therapies [32]; (2) antibodies against IFN- $\alpha$  are found in some MG patients, mainly those with thymoma [33, 34]; and (3) a thymic transcriptome analysis of different MG patient subgroups shows a significant increase in the expression of IFN-I-induced genes [35, 36]. IFN-I can directly affect TEC behavior but also thymocyte

differentiation/activation. A study on peripheral blood mononuclear cells (PBMCs) demonstrates that a chronic elevation of IFN- $\alpha$  can modify the effector/regulatory T cell ratio toward effector T cells and autoimmunity [37].

Different IFN-I and IFN-III subtypes, but especially IFN- $\beta$ , are overexpressed in the thymus of MG patients [38]. Analyzing in detail the *in vitro* effect of IFN- $\beta$ , it is shown that IFN- $\beta$  induces specifically the expression of  $\alpha$ -AChR and not that of other TSAs. It also increases TEC death and the uptake of TEC proteins by DCs. In parallel, IFN- $\beta$  triggers the expression of the chemokines CXCL13 and CCL21 by TECs and lymphatic endothelial cells, respectively. The B cell-activating factor (BAFF), which favors B cell survival, is overexpressed by TECs in MG thymus and is also induced by IFN- $\beta$  in TEC cultures. Similar changes are observed *in vivo*. Polyinosine-polycytidylic acid (poly(I:C)) is a synthetic molecule mimicking double-stranded RNA (dsRNA) from viral infections. The injections of poly(I:C) to C57BL/6 mice trigger the thymic overexpression of IFN- $\beta$  and IFN- $\alpha$ 2 associated with increased expressions of CXCL13, CCL21, and BAFF and favor the recruitment of B cells. These changes are not observed in the thymus of IFN-I receptor knock-out (KO) mice injected with poly(I:C), even if IFN- $\beta$  and IFN- $\alpha$ 2 are overexpressed [39]. Altogether, these results demonstrate that IFN- $\beta$  could play a central role in thymic events leading to MG by triggering the overexpression of  $\alpha$ -AChR probably leading to thymic DC autosensitization, the abnormal recruitment of peripheral cells and GC formation as summarized in Fig. 1.

The critical role played by inflammation in thymic changes associated with EOMG is also put forward thanks to a new study using a novel transgenic mouse model with a thymic overexpression of CXCL13. It is demonstrated that CXCL13 overexpression by itself is not sufficient to induce peripheral B-cell recruitment to the thymus. However, upon poly(I:C) injections leading to thymic inflammation, CXCL13 triggered a strong recruitment of B cells to the thymus [40].

IFN-I subtypes may also play a central role in thymoma-associated MG. Indeed, huge increases of IFN- $\alpha$ 2, IFN- $\alpha$ 8, IFN- $\omega$ , and to a lesser extent of IFN- $\beta$  are detected in thymoma-associated MG but not in thymoma without MG or in control thymuses. These results reinforce again a specific role of IFN-I and the anti-AChR response associated with MG [41]. The inflammatory environment characteristic of MG thymus, with IFN-I overexpression, could act at different thymic levels, the AChR expression by TECs, the sensitization against  $\alpha$ -AChR, and consequently triggers an autoimmune response against AChR (Fig. 1).

### Activation of Innate Immunity Pathways in MG Patients

Autoimmune diseases are multifactorial diseases, and in the last decade, diverse environmental factors have been singled

out to explain the emergence of these diseases [42]. Pathogens are major environmental-factor candidates for driving/perpetuating autoimmunity, but it is difficult to link infections with the onset of the disease [43]. Indeed, symptoms related to autoimmune diseases occur probably long after the pathogen infection, when the pathogen might have already been cleared or the antiviral immune responses might have subsided (“hit-and-run” hypothesis) [43]. Nevertheless, accumulating evidences tend to support the contribution of pathogens in promoting, exacerbating, or maintaining autoimmune conditions.

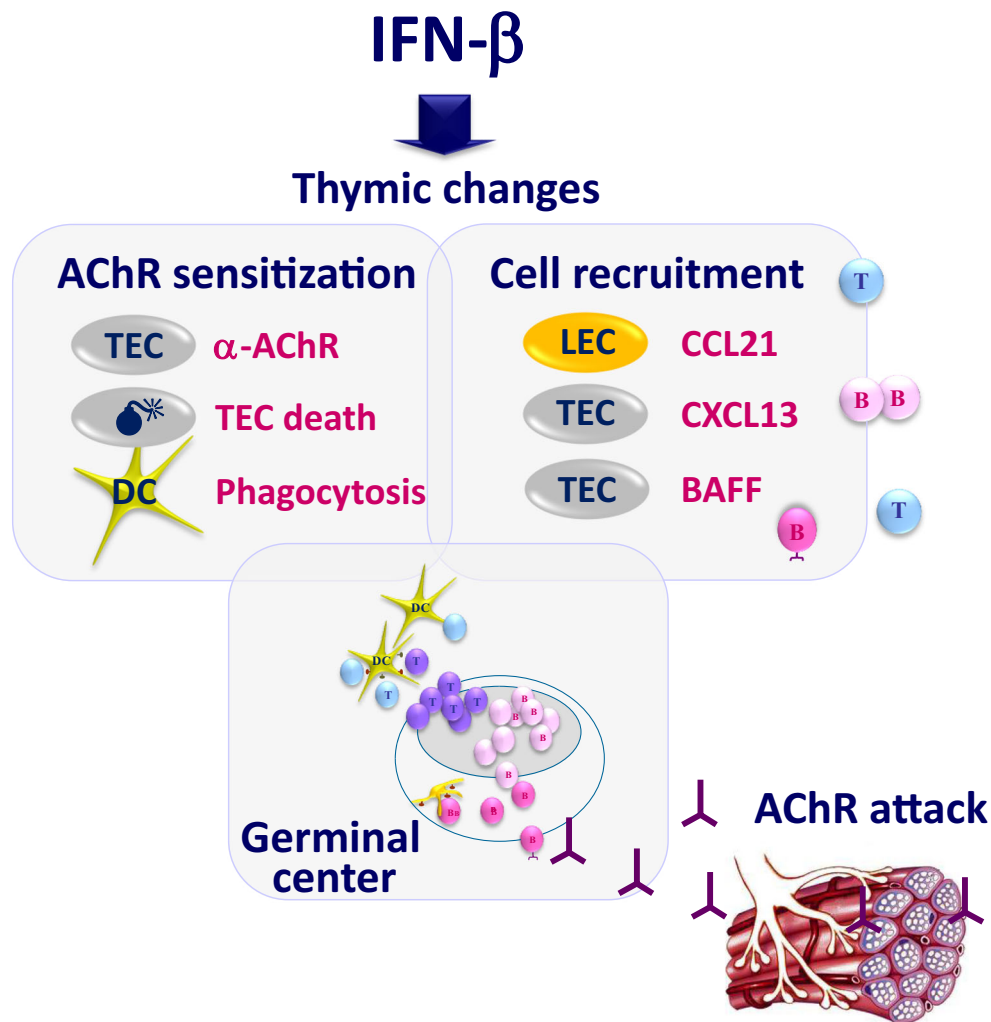
The thymus is a common target organ for infectious diseases [44]. Striking evidence of chronic inflammation and emerging data on persistent viral infections in the thymus of MG patients strongly support the hypothesis that the activation of the innate immune system may trigger or favor MG development. Bernasconi et al. describe poliovirus-infected macrophages and the presence of Epstein-Barr virus (EBV)-infected B cells in MG thymus [45, 46, 47]. The ability of EBV to infect and transform B cells, allowing them to evade immune surveillance [47], makes it a prime suspect for chronic infection and inflammation. EBV could activate and immortalize intrathymic autoantibody-producing cells and sustain chronic inflammation via toll-like receptor (TLR)-mediated pathways [48].

As we observed in the MG thymus an anti-viral signature together with an increased expression of IFN-I-induced genes [29], we suggest that inappropriate activation of pathogen-signal pathways could be involved in thymic changes associated with MG.

## TLR: Sensors of Pathogen Infections and Implication in Autoimmune MG

### TLR Signaling

A major role in innate immunity is played by TLRs, a type of pattern recognition receptors (PRRs) able to recognize conserved microbial molecules. Each TLR recognizes specific microbial derived molecular structures [49]. Of the TLR family, 11 and 12 members have been identified in human and mouse, respectively. TLRs are essentially described in immune cells but can be found in various other cell types. TLR activation and signaling are largely described in diverse reviews [50, 51, 52]. Briefly, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located on the cell surface and especially recognize microbial membrane components. TLR3, TLR7, TLR8, TLR9, and TLR11 are mainly located on the intracellular endosome membranes. Intracellular TLRs recognize nucleic acids derived from bacteria or viruses (Table 1). TLRs are delivered from the endoplasmic reticulum to the endosome by the endoplasmic reticulum-resident membrane protein UNC93B1.



**Fig. 1** Central role of IFN- $\beta$  in thymic changes associated with MG. IFN- $\beta$  induces the expression in TECs of  $\alpha$ -AChR, the main autoantigen in MG. This effect is very specific, as IFN- $\beta$  does not induce the expression of other AChR subunits or other tissue-specific antigens. IFN- $\beta$  also induces TEC death and the uptake of TEC proteins by DCs, suggesting a potential sensitization of DCs to the  $\alpha$ -AChR [39]. In parallel, IFN- $\beta$  increases the expression of the chemokines CXCL13 and CCL21 by TECs and lymphatic endothelial cells, respectively [39]. These two

chemokines are involved in GC development and are overexpressed in MG with follicular hyperplasia [28, 13]. The B cell-activating factor (BAFF), which favors autoreactive B cells, is overexpressed by TECs in MG thymus and is also induced by IFN- $\beta$  [39]. Altogether, these results demonstrate that IFN- $\beta$  plays a central role in thymic events leading to MG by triggering the overexpression of  $\alpha$ -AChR, probably leading to thymic DC auto-sensitization against AChR and the abnormal recruitment of peripheral cells involved in GC formation

The interaction with their ligands leads to TLR dimerization and internalization. Except TLR3, all TLRs interact with the intracellular adaptor MyD88 (myeloid differentiation primary response gene 88) and activate downstream signaling cascades. TLR3, but also TLR4, activates intracellular signaling pathways via TIR domain-containing adapter-inducing interferon- $\beta$  (TRIF). TLR2, TLR5, and TLR9 have also been described as being able to activate TRIF signaling pathway [53, 54, 55]. In all cases, TLR activation leads to the stimulation of the NF- $\kappa$ B, p38, JNK, and/or IFN-regulatory factor (IRF) signaling pathways, resulting in the production of inflammatory cytokines and in particular IFN-I [56]. The

activation of TLR signaling pathways is probably more complex than what has been so far described as TLRs are able to interact and form heterodimers that could enhance, inhibit, or modulate more specifically the cell response according to the pathogens present in the local environment [50].

A lower amount of intracellular TLRs are also localized on the cell surface. In the case of TLR3, Pohar et al. demonstrate that the UNC93B1 protein promotes the translocation of a differentially glycosylated form of TLR3 to the cell surface. In addition to the translocation, UNC93B1 is upregulated upon poly(I:C) treatment and enhances the responsiveness of TLR3 and TLR9 by increasing their lifetime. They

**Table 1** List of human TLRs

TLRs	Ligands	Cellular localization	Interaction with other TLRs	First adaptor for signalization	References
TLR1	Triacyl lipoproteins from bacteria	Cell surface	TLR2 TLR4	MyD88	[126]
TLR2	Microbial molecules from bacteria <i>Mycoplasma</i> and yeast	Cell surface	TLR1 TLR6 TLR10	MyD88 TRIF	[127, 55]
TLR3	dsRNA from viruses	Endosome cell surface		TRIF	[128, 57, 129]
TLR4	Lipopolysaccharide from Gram-negative bacteria Mannans from yeast Glycoinositolphospholipids from the protozoan <i>Trypanosoma cruzi</i> Respiratory syncytial virus fusion protein	Cell surface	TLR1 TLR5	MyD88 TRIF	[98, 130]
TLR5	Flagellin from bacteria	Cell surface	TLR4	MyD88 TRIF	[131, 53]
TLR6	Diacyl lipoproteins from bacteria <i>Mycoplasma</i>	Cell surface	TLR2	MyD88	[126]
TLR7	ssRNA from viruses	Endosomes		MyD88	[128, 58]
TLR8	ssRNA from viruses	Endosomes		MyD88	[59, 132]
TLR9	Unmethylated CpG oligonucleotide DNA from viruses and bacteria	Endosome cell surface		MyD88 TRIF	[133, 54, 60]
TLR10	Functional RNA-protein complex of influenza virus	Cell surface	TLR2	MyD88	[134, 135]
TLR11	Flagellin from bacteria Profilin-like molecule from <i>Toxoplasma gondii</i>	Endosomes		MyD88	[136]

hypothesize that TLR3 on cell surface could allow the detection of low amount of circulating dsRNA and potentiate the activation of TLR9 through the upregulation of UNC93B1 [57]. The other endosomal TLRs are also detected on the cell surface, and for TLR9, it seems to act as a negative regulator of the B cell response [58, 59, 60].

The first function of TLRs is to set up an innate immune response to protect the organism from pathogens. However, studies, both in experimental settings and in a number of human autoimmune diseases, indicate that the loss of regulation of TLR signaling can lead to chronic inflammation and auto-sensitization, thereby promoting or favoring inflammatory and autoimmune diseases [61, 51]. The contribution of TLRs in autoimmunity can mainly be explained by the ability of these receptors to stimulate the maturation of antigen-presenting cells, the production of IFN-I, and of other pro-inflammatory cytokines [49]. TLR3, TLR4, TLR7, TLR8, and TLR9 activation promotes Th1-type immune responses, while signaling via TLR2 (along with TLR1 or TLR6) and TLR5 favors Th2-type immune responses [62]. TLR ligands also influence the development of regulatory T cells [63] and Th17 cells. Moreover, along with antigen binding to the B cell receptor and CD40 stimulation, TLR stimuli, mainly those mediated by TLR3, TLR7, and TLR9, provide additional costimulatory signals for proliferation, maturation, and survival of B cells, including autoreactive B cells, thus compromising B cell tolerance [64], [65, 66]. In vitro and in vivo studies suggested that persistent activation of nucleic acid-sensing TLRs, such as TLR7, TLR8, and TLR9, may cause systemic

autoimmunity, through stimulation of B cell activation and autoantibody production [67].

#### Abnormal TLR Activation in PBMCs of MG Patients

Abnormal expression of TLRs on PBMCs has been demonstrated in various autoimmune diseases. Wang et al. analyzed TLR messenger RNA (mRNA) expression in PBMCs from MG patients. While TLR7 expression is similar in MG and controls, TLR1, TLR6, and TLR10 are significantly downregulated and TLR2, TLR3, TLR4, TLR5, TLR8, and TLR9 are significantly upregulated in PBMCs from MG compared to healthy donors [68]. In the classical experimental autoimmune MG (EAMG) model, TLR4 expression is also found increased in AChR-specific B cells [69]. Of interest, the expression of TLR9 is positively correlated to the quantitative MG score (QMG) of patients. However, they do not observe a normalization of TLR expression in patients under methylprednisone treatment [68]. These changes in TLR expression could reflect changes in the percentage of specific peripheral cells in MG patients or a different activation state for PBMCs.

#### Abnormal TLR Expression in MG Thymus

The expression level of TLRs in the thymus of MG patients has been analyzed in different studies. These global analyses reflect the implication of all thymic cells and do not detail which cells are implicated. Thymic stromal cells such as TECs and myoid cells express all TLRs [38]. In periphery,



immune cells express all TLRs at various degrees [70], but the expression of TLRs by thymocytes themselves has never been fully investigated.

By semiquantitative PCR measures, Bernasconi et al. first demonstrate the overexpression of TLR4 in MG thymus characterized by B-cell infiltrations but not in hyperplastic MG thymuses. They do not observe changes for TLR2, TLR3, and TLR5 expressions, but three quarters of the MG patients included in the study were receiving immunosuppressive therapies that might affect the global level of TLR expression [71]. In another study, we demonstrate the overexpression of TLR3 in the thymus of MG patients [38] and no difference is observed between patients with a high or low degree of thymic hyperplasia, suggesting that the higher TLR3 expression level is not linked to the increased number of B cells in hyperplastic thymuses. In parallel, we observed an increase in TLR3 expression in TEC cultures derived from the thymus of MG patients compared to controls (data not shown). This increased expression of TLR3 in MG thymus could reflect an abnormal activation subsequent to a viral infection. Moreover, TLR3 expression is detected in TECs and could be involved in the overexpression of IFN- $\beta$  in MG thymuses. In the same study, an increased expression of the protein kinase R (PKR), another dsRNA-interacting protein, but not for RIG1 and MDA5 helicases, is measured in MG thymuses [38]. Recently, Cavalcante et al. demonstrated the overexpression of TLR7 and TLR9 in the thymus of MG patients. By immunohistochemistry, a stronger expression of TLR7 and TLR9 is observed in B cells, TECs, plasmacytoid dendritic cells, and macrophages in MG thymuses compared to controls. TLR7 is also increased in thymic myeloid dendritic cells, and its transcriptional levels positively correlate with those of IFN- $\beta$  [72].

In order to have a global view on the expression levels of all TLRs in the thymus of MG patients, we carried out quantitative PCR on control thymuses compared to thymuses from MG patients with various degrees of thymic hyperplasia (Fig. 2a). First, we observed that TLR1 to TLR9 were all expressed in control female thymuses with TLR1 and TLR9 being the most expressed and TLR8, TLR5, and TLR2 the less. We did not observe significant changes for TLR1, TLR2, TLR5, and TLR7 expressions in untreated MG patients and controls. We confirmed the increased expression level of TLR3 for all MG patients [38]. We observed an increased expression for TLR4 but only in hyperplastic thymuses. For TLR3 and TLR4, the expression levels were even exacerbated in patients under corticoid treatment. We also demonstrated a specific overexpression for TLR6, TLR8, and TLR9 in hyperplastic MG thymuses, suggesting a link with the presence of GCs, especially as the levels of expression were lowered in patients with corticoid treatments. Indeed, corticoids are known to decrease the number of GCs in MG thymuses [28]. Moreover, by analyzing the level of CD19 mRNA

expression in these thymic biopsies, a significant correlation was observed between the level of CD19 mRNA expression and TLR6, TLR8, and TLR9 mRNA but not for TLR3 and TLR4 mRNA (Fig. 2b).

To investigate the potential consequences of pathogen infection on the thymus, our team analyzed the effects of TLR agonists (from TLR1 to TLR9) on human TECs in culture. We demonstrated that poly(I:C), a synthetic analog of dsRNA, specifically induces the thymic overexpression of  $\alpha$ -AChR but not other AChR subunits or TSAs. This induction is mediated through TLR3, PKR, and by the release of IFN- $\beta$  but is completely inhibited by IFN-I receptor or IFN- $\beta$ -blocking antibodies [38].

If poly(I:C) seems to specifically affect AChR expression by TECs, the activation of other TLR signaling pathways could also be crucial in sustaining the inflammatory response leading to MG development and the chronicity of the disease. The thymic EBV signature observed in MG thymuses could explain the activation of diverse TLR signaling pathways. Indeed, EBV dsDNA and RNA could account for TLR9 and TLR7 activation [73]. EBV also encodes small RNAs with a dsRNA-like structure (EBER) that can trigger TLR3 signaling [74]. TLR2 is also suspected to play an important role in immune responses directed against EBV infection [75], but TLR2 expression was not upregulated in MG patients (Fig. 2a). Moreover, TLR7 activation can increase the expression of EBV latent membrane protein 1 (LMP1) in EBV-infected cells and potentiates those cells for production of IFN-I by TLR3 or TLR9 activation [76].

## Role of TLR Agonists in Experimental Autoimmune Models: Focus on Mouse MG Models

### The EAMG Model Based on AChR Immunizations Using Complete Freund's Adjuvant

In almost all induced experimental models for autoimmune diseases, a purified antigen is injected emulsified in complete Freund's adjuvant (CFA; Table 2). EAMG can be triggered by active immunization with AChR peptides, AChR subunits, recombinant fragments of muscle AChR, or purified AChR isolated from electrical organs of torpedo fish (T-AChR) [77]. A guideline review has been recently published, detailing thoroughly the mouse EAMG model [78]. Briefly, strains carrying the H-2b haplotype are more susceptible to EAMG and the C57BL6 mouse strain is the conventional wild-type mouse strain used in most studies. For immunization, 20–30  $\mu$ g of T-AChR emulsified in CFA containing heat-killed and dried mycobacterium tuberculosis (MTB) is used [79]. A small volume of the emulsion is subcutaneously injected in several sites. For the first immunization, the injections are done in the hind foot pads and in four to six sites on the back including the base

of the tail. After 25–30 days, mice should be immunized a second time with the T-AChR in CFA or IFA (incomplete Freund's adjuvant) emulsions but only in sites on the back. After the second immunization, around 50–60 % of the mice develop EAMG symptoms. If the EAMG incidence is less than 50 %, a third immunization with T-AChR in IFA can be done [78]. EAMG is easier to induce in rats as one immunization is sufficient in inducing strong MG symptoms [80].

As for many autoimmune experimental models, the use of CFA is mandatory to set up these disease models and MTB is critical in the activation of innate immune signaling pathways, leading to the sensitization against the injected antigen. How CFA acts is not clearly defined even if it has been used since the 50s. CFA is composed of IFA (a paraffin oil) with added MTB. It is generally assumed that administering a protein antigen as a water-in-oil emulsion prolongs its lifetime at sites of injection, leading to the formation of local and distant granulomas composed of mononuclear phagocyte cells and oil drops. Then, a strong and long-lasting inflammatory reaction develops at sites of injection but also in draining lymph nodes. IFA on its own induces a Th2-dominated response compared to CFA which induces a Th1-dominated response [81]. CFA induces the expression of pro-inflammatory cytokines, mainly tumor necrosis factor- $\alpha$ , interleukin (IL)-12, IL-6, IFN- $\gamma$ , and chemokines by mononuclear phagocyte cells and natural killer cells. The released cytokines ultimately lead to polyclonal activation of T cells and ultimately antigen-specific T cells. In the EAMG mouse model, CFA involves both a strong Th1 and a Th2 response, but also, B cell growth and differentiation associated with the secretion of IgG2 and IgG1 antibodies [82]. The IFA response in mice favors the production of IgG1 antibodies that do not bind the complement. In contrast, the predominant Th1 response induced by CFA favors the production of complement-binding antibody isotypes, such as IgG2b and IgG2c in C57BL/6 mice, that can be pathogenic and in the case of anti-AChR antibodies cause the destruction of AChR at the neuromuscular junction [83].

Heat-killed and dried MTB contained in CFA is central in inducing the sensitization against the injected antigen. MTB is responsible for tuberculosis. It is a pathogenic bacteria infecting and surviving in mononuclear cells. TLR2, TLR4, TLR9, and possibly TLR8 are able to interact with MTB pathogen patterns. TLR activation upregulates the transcription of pro-inflammatory cytokines, which are essential for the recruitment of immune cells to the site of infection and the control of MTB infection. As a first line of defense, MTB infection triggers an innate immune response followed by an adaptive immune response to contain the infection. This is characterized by the development of antigen specific CD4<sup>+</sup> T cells that secrete Th1 pro-inflammatory cytokines such as IFN- $\gamma$  and the activation of CD8<sup>+</sup> T cells. Th1 and Th17 pro-inflammatory cytokines are most probably central to the development of the immune response against MTB and the

development of autoimmune experimental models. Indeed, IFN- $\gamma$  KO and IL-17 KO mice are resistant to EAMG [84, 85, 86].

Even if it is evident that MTB infection activates TLR signaling pathways, the activation of these pathways by CFA itself is not completely proven. Gavin et al. showed that CFA-enhanced antibody responses occur in MyD88 and TRIF (Trif Lps2/Lps2 mice) KO mice, which cannot transmit signals through TLRs [87]. However, numerous studies have proven that MyD88 KO mice are less susceptible to experimental autoimmune models [88, 89, 90]. Specific TLR KO mice do not seem to be resistant to the induction of autoimmune experimental models, and it was suggested by Fang et al. that TLR2, TLR3, TLR4, and TLR9 are highly redundant in transducing CFA adjuvant effect to induce the experimental autoimmune models [88, 91].

### Alternative Use of Other Adjuvants to Induce EAMG

Different attempts have been made to use other adjuvants than CFA for the EAMG model. Indeed, only 50–60 % of the mice develop MG symptoms and CFA injections in foot pads can induce a local pain and consequently restrictions of national regulatory authorities. The induction of EAMG in mice without CFA was tried with the intraperitoneal injection of affinity-purified AChR prepared from the BC3H1 cell lines. MG symptoms were observed but appear less effective, and this method was not used afterward [92, 93].

Milanui et al. describe a model where aluminum hydroxide is used instead of CFA to induce MG in C57BL/6 mice. Aluminum hydroxide is used for human vaccination. Its efficacy resides in its ability to induce the release of uric acid that is considered as a danger signal by the immune system. Aluminum hydroxide leads thus to the activation of monocyte-derived inflammatory cytokines improving the uptake of the antigen. Aluminum hydroxide induces mainly a Th2 response associated with IgG1 production [94]. Compared to mice immunized with T-AChR/CFA and boosted in T-AChR/IFA, mice immunized several times with T-AChR/aluminum hydroxide develop milder EAMG symptoms and lower levels of anti-T-AChR or mouse-AChR antibodies. With aluminum hydroxide, mice produce essentially Th2 cytokines and anti-T-AChR IgG1 that are less pathogenic as they do not bind the complement. In fact, this study even demonstrates that anti-T-AChR CD4<sup>+</sup> T and B cells with a Th2 phenotype may cooperate in protecting mice from EAMG [82]. A similar observation was made by Oshima et al. that used an AChR peptide solubilized in aluminum hydroxide and killed pertussis organisms to vaccinate and protect C57BL/6 mice prior immunization with T-AChR in CFA [95].

A new adjuvant formulation was developed in the 90s as an alternative to CFA for producing antisera in animals. This

adjuvant named Titermax is also a water-in-oil emulsion composed of a block copolymer, squalene (a metabolizable oil), and microparticulates. It has a lower toxicity than CFA and does not contain microbial products [96]. The use of this adjuvant for the induction of autoimmune experimental models is hardly described. Nevertheless, Titermax has been tested for its ability to induce EAMG. C57BL/6 mice were inoculated with T-AChR emulsified in Titermax. Mice immunized displayed characteristic MG muscle weakness with electrophysiological defect, elevated serum anti-AChR antibodies, and muscle AChR loss [97]. However, this new adjuvant was not used afterward.

### Alternative Use of Lipopolysaccharide Instead of CFA to Induce EAMG

Bacterial lipopolysaccharides (LPSs) are present on the surface of almost all Gram-negative bacteria and act as extremely strong stimulators of innate immunity. LPSs activate intracellular pathways through TLR4. TLR4 is expressed by various cells types and not only by immune cells. It is the only TLR that does not directly bind its ligand, and LPS stimulation of TLR4 includes the participation of other molecules. LPS first binds to the LPS-binding protein (LBP) and can then interact with CD14 and subsequently with the TLR4/MD-2 receptor complex (TLR4 being a transmembrane protein and MD-2 a soluble protein). CD14 is a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form. If CD14 is mainly known to be expressed by macrophages, it can also be expressed at a lower level by cells of both hematopoietic and non-hematopoietic origin as a cell membrane or secreted proteins. Soluble CD14 could even allow the activation of TLR4 by LPS in CD14 negative cells. Ultimately, binding of LPS to CD14/TLR4/MD2 results in dimerization of the receptor complex and the activation of different signaling pathways is elicited through MyD88 and TRIF molecules [98, 99]. LPS effects through TLR4 activation is suspected to play a role in autoimmune diseases [100].

Allman et al. have used LPS as adjuvant instead of MTB in CFA to induce the EAMG model [101]. C57BL/6 mice were immunized by several subcutaneous injections in foot pads and shoulders with T-AChR and LPS emulsified in IFA or T-AChR emulsified in CFA. Mice were immunized three times at days 0, 28, and 56. AChR-LPS/IFA-immunized mice develop clinical signs that are similar to those observed with the AChR/CFA immunization. They produce anti-AChR antibodies, and IgG2 and C3 deposits are detected at the neuromuscular junction. However, B cell activation leading to the production of the anti-AChR antibodies is different in AChR-LPS/IFA and AChR/CFA models. Using CD4<sup>-/-</sup> KO mice, they demonstrated that CFA immunization requires CD4 costimulation of B cells to induce anti-AChR IgG2 secretion while LPS immunization does not.

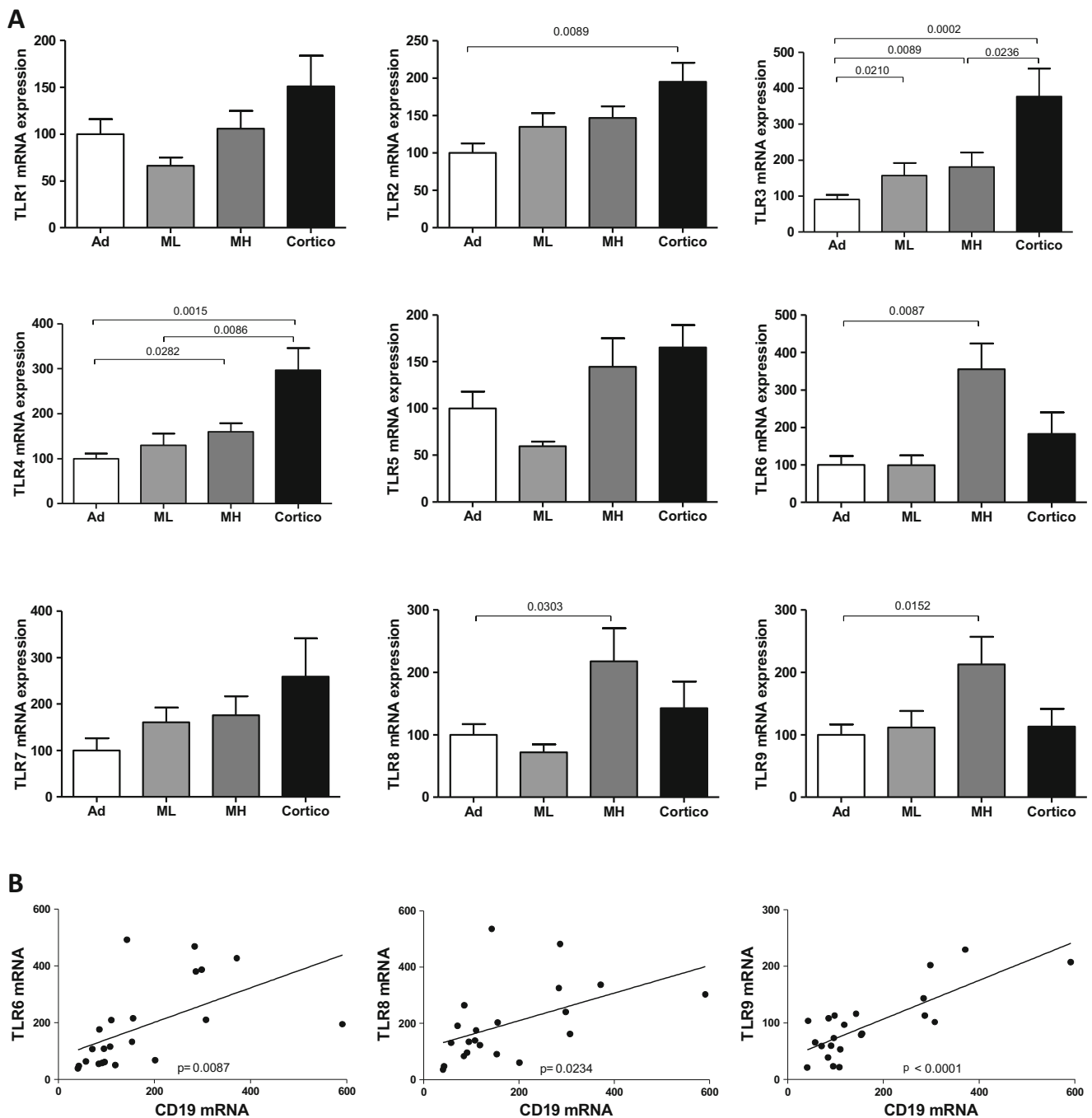
**Fig. 2** Analysis of TLR mRNA expression in the thymus of MG patients. Thymic fragments were obtained from MG female patients (15–44 years old) after thymectomy or from adult females (15–30 years old) undergoing cardiovascular surgery at the Marie Lannelongue Chirurgical Center (Le Plessis Robinson, France). MG patients were all anti-AChR positive treated by anti-cholinesterase drugs but had no other known diseases (including thymoma). Thymuses were classified as follows: non-MG adults (Ad,  $n=6-13$ ), MG patients with low thymic hyperplasia (ML; with two or fewer GCs per section,  $n=6-13$ ) or high thymic hyperplasia (MH; with three or more GCs per section,  $n=6-13$ ), or corticosteroid-treated MG patients (Cortico,  $n=6-11$ ). All the studies on thymuses were approved by the local Ethics Committee (CCP agreement no. C09-36-France). Total RNA was extracted, reverse-transcribed, and analyzed by real-time PCR on the LightCycler 480 system as previously described [39]. The primer used were as follows: *TLR1* (F 5' GGGTCAGCTGGACTTCAGAG 3', R 5' AAAATCCAAA TGGAGGAACG 3'), *TLR2* (F 5'GGGTTGAAGCACTGGACAAT 3', R 5'TCCTGTTGTTGGACAGGTCA 3'), *TLR3* (F 5'AGCCTTCAA CGACTGATGCT 3', R 5'TTTCCAGAGCGGTGCTAAGT 3'), *TLR4* (F 5'TGAGCAGTCGTGCTGGTATC 3', R 5'CAGGGCTTTTC TGAGTCGC 3'), *TLR5* (F 5'AAAAATGGGATGGTCCATGA 3', R 5'TGGAGAAGCCGAAGGTAAGA 3'), *TLR6* (F 5' TAAATTGGACTGGCCTGGAC 3', R 5'GGCCGAAACTG GTTATTGA 3'), *TLR7* (F 5'CCTTGAGGCCAACACATCT 3', R 5' GTAGGGACGGCTGTGACATT 3'), *TLR8* (F 5'CAGAGCATC AACCAAAGCAA 3', R 5'CTGTAACACTGGCTCCCAGCA 3'), *TLR9* (F 5'CAGCAGCTCTGCAGTACGTC 3', R 5'AAGGCCAGGTA ATTGTCACG 3'), *CD19* (F 5'TACTATGGCACTGGCTGCTG 3', R 5' CACGTTCCCGTACTGGTTCT 3'), and *28S* (F 5' GGTAGGGACA GTGGGAATCT 3', R 5'CGGGTAAACGGCGGGAGTAA 3'). **a** mRNA expression level for TLRs in the thymus. mRNA expression was normalized to 28S RNA. *p* values were assessed by the Mann-Whitney test, and *p* values <0.05 are indicated. **b** Correlations of CD19 mRNA expression and TLR mRNA expression in all thymic samples. The *p* values were obtained by the non-parametric correlation test (Spearman's test)

Rose and collaborators also investigated the effect of LPS in the induction of the experimental thyroiditis model classically based on active immunization of mice with thyroglobulin emulsified in CFA. They observed that intravenous injections of LPS, in parallel to thyroglobulin injections, are sufficient to induce thyroglobulin-specific autoantibodies and inflammatory lesions in the thyroid gland [102]. Damotte et al., also demonstrate in the experimental autoimmune thyroiditis model that while CFA pathogenic effects are dependent on CD8 T cells, LPS effects are independent of CD8 T cells. LPS seems to enhance the immunogenicity of autoantigens but also to affect directly thyrocytes by inducing the expression of chemokines that could facilitate its lymphocytic infiltrations [103].

### A Novel Experimental MG Model Based on Poly(I:C) Injections

Poly(I:C) that mimics dsRNA from viral infections is well known to interact with TLR3 but also PKR and RNA helicases (MAD5 and RIG-I) [104]. It has been demonstrated that dsRNA signaling induced by poly(I:C) specifically





triggers the overexpression of  $\alpha$ -AChR in human TECs and not the expression of other TSAs expressed by TECs. This induction is mediated through TLR3 and PKR and by the release of IFN- $\beta$ . Interestingly, the overexpression of TLR3, PKR, IRF-7, IRF-5, and IFN- $\beta$ , which are all involved in antiviral responses induced by dsRNA, are also observed in the MG thymus [38].

On the basis of these observations, C57BL/6 mice were injected with 200  $\mu$ g of poly(I:C) three times a week. After 1 week, specific changes are observed in the thymus of poly(I:C)-injected mice, increased expression of IFN- $\beta$ ,

IFN- $\alpha$ 2, and  $\alpha$ -AChR but also CXCL13 and CCL21 which are overexpressed in the MG thymus [28, 13]. In parallel, a recruitment of B cells is observed after poly(I:C) injections. These thymic changes are not observed in IFN-I receptor KO mice, underlying the central role played by IFN-I in thymic changes associated with MG [38, 39].

After 6 weeks, poly(I:C) induces in mice clinical and biological signs of MG: (1) using a grip strength apparatus, which is a global test of fatigability, poly(I:C)-injected mice were weaker compared to control mice. (2) Poly(I:C) triggers the production of anti-AChR antibodies, and B cells purified from

**Table 2** Experimental mouse models for autoimmune diseases using CFA to induce an active immunization against a specific antigen

Autoimmune disease	Induced experimental mouse model	Antigen used for immunization	Main adjuvant	References
Rheumatoid arthritis	Collagen-induced arthritis (CIA)	Collagen II	CFA	[137]
Multiple sclerosis	Experimental autoimmune encephalomyelitis (EAE)	Proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG)	CFA	[138]
Myasthenia gravis	Experimental autoimmune myasthenia gravis (EAMG)	Acetylcholine receptor (AChR)	CFA	[78]
Myositis	Experimental autoimmune myositis (EAM)	Myosin	CFA	[139]
Autoimmune inflammatory heart disease	Experimental autoimmune myocarditis (EAM)	$\alpha$ -myosin heavy chain peptide	CFA	[89]
Autoimmune hepatitis	Experimental autoimmune hepatitis (EAH)	Syngeneic S-100 antigen	CFA	[140]
Autoimmune uveitis	Experimental autoimmune uveitis (EAU)	Interphotoreceptor retinoid-binding protein (IRBP)	CFA	[91]
Primary biliary cholangitis	Murine autoimmune cholangitis (EAC)	2-octynoic acid (2-OA)	CFA	[141]
Glomerulonephritis and vasculitis	Experimental autoimmune vasculitis (EAV)	Myeloperoxidase (MPO)	CFA	[142]
The antiphospholipid syndrome	Experimental antiphospholipid syndrome (EAPS)	$\beta$ 2-glycoprotein I ( $\beta$ 2-GPI)	CFA	[143]
Hashimoto's thyroiditis	Experimental autoimmune thyroiditis (EAT)	Thyroglobulin (Tg)	CFA	[103]

lymph nodes proliferate more strongly in the presence of AChR in vitro. (3) AChR density on mouse diaphragm muscle is decreased in poly(I:C)-injected mice, suggesting a partial loss of AChR on diaphragm muscle that could be due to the destruction of AChR by anti-AChR antibodies [38].

Compared to the classical EAMG model, clinical signs are more subtle but all mice are almost similarly affected. The levels of anti-AChR antibodies are lower, but they are specifically directed against the mouse AChR and could be more efficient in targeting AChR on mouse muscle. In contrast, in the EAMG model, anti-AChR antibodies are much higher but these antibodies are mainly directed against the T-AChR, and the titer against mouse AChR ranges only between 0.2 and 2 % of the titer for anti-T-AChR [105].

In poly(I:C)-injected mice, the thymic changes occurring after 1 week are not observed anymore after 6 weeks, but the initial changes associated with the recruitment of B cells could be sufficient to trigger a sensitization against  $\alpha$ -AChR. These autoreactive B cells could afterward migrate to the periphery as they do in human MG. Indeed, Fujii et al. demonstrated that autoreactive B cells are found in lymph nodes of MG patients [106]. In poly(I:C)-injected mice,  $\alpha$ -AChR autoreactive B cells are also present in lymph nodes. Nevertheless, in contrast to the human pathology, no thymic GCs are found even after prolonged injections of poly(I:C). It is clear that the genetic background involved in human MG is not recapitulated in mice, and this could explain their ability to overcome initial thymic changes.

This study shows that MG symptoms can be induced in mice without active immunization with T-AChR or AChR peptides. Indeed, dsRNA signaling activation reflects a pathogen infection that could induce an autoimmune response against AChR. Poly(I:C) injections on their own have also been shown to favor the development of other experimental autoimmune models in genetically susceptible mouse strain. Poly(I:C) treatment induces local joint inflammation as in arthritis, if administered directly into the joints of healthy mice [107]. It can induce an early development of primary biliary cirrhosis-like cholangitis in C57BL/6 mice [108] and of pancreatitis in MRL/Mp mice [109]. Poly(I:C) aggravates lupus nephritis in MRL lpr/lpr mice [110, 111] and accelerates the development of salivary gland disease in NZB/WF1 mice, a strain susceptible to Sjögren's syndrome disease development [112].

In parallel, poly(I:C) has also been used to boost the adjuvant effect of CFA or IFA in classical autoimmune experimental models. Simultaneous administration of poly(I:C) in BALB/c mice immunized with an insulin peptide in IFA induces insulinitis [113]. Poly(I:C) injections exacerbate the retinal autoimmunity model induced by the immunization of mice with the retinal autoantigen interphotoreceptor retinoid-binding protein (IRBP) in CFA [114]. Poly(I:C) also aggravates the autoimmune cholangitis model induced by 2-

octynoic acid injections in CFA [115]. Generally, poly(I:C) exacerbates the symptoms but it can also suppress relapsing demyelination in a murine experimental autoimmune encephalomyelitis model via the induction of IFN- $\beta$  [116]. This specific improving effect of poly(I:C) in the encephalomyelitis model is probably related to the fact that in contrast to many other autoimmune diseases, IFN-I plays a neuroprotective role in the central nervous system [117]. Moreover, IFN- $\beta$  is beneficial for multiple sclerosis patients and used as a treatment [118].

Experiments are also going on in our laboratory to investigate the effects of poly(I:C) injections in the classical EAMG model in an attempt to increase the percentage of mice with MG symptoms and develop a MG mouse model with thymic hyperplasia. Preliminary experiments are very encouraging as almost all animals get sick (unpublished data).

### Improving MG Experimental Models

The classical EAMG model obtained with T-AChR immunization in CFA has been used for more than 40 years in research laboratories to improve the understanding of the human disease and also to test different therapeutic approaches [119, 120, 78]. The rat model is more sensitive to T-AChR immunization as one injection is sufficient to trigger MG symptoms. The mouse model is more challenging but is very convenient to test transgenic mice. These models are perfectly relevant to study the consequences of the autoimmune attack of AChR. Unfortunately, mouse or rat models do not present thymic abnormalities as observed in the human disease [121]. Thymectomy of rats with EAMG does not improve either the evolution of the disease [122]. These observations indicate that thymic abnormalities observed in human MG do not result from the production of anti-AChR antibodies in the periphery but rather suggest the role of the thymus as the effector organ in the human pathology.

Abnormal overexpression of CXCL13 is observed in many inflamed tissues, in particular in autoimmune diseases. Thymic hyperplasia observed in EOMG patients is characterized by ectopic GCs and is correlated with high levels of anti-AChR antibodies [6]. The B cell chemoattractant, CXCL13, is overexpressed by TECs in EOMG patients and suspected to trigger B cell recruitment in MG [28]. A novel transgenic mouse with a keratin 5-driven CXCL13 expression has been created by Weiss and collaborators. These mice display a clear thymic overexpression of CXCL13 and have been used for the classical EAMG model. These transgenic mice are clearly more susceptible to EAMG and display stronger clinical signs, higher titers of anti-AChR antibodies. Moreover, in the thymus, a recruitment of B cells is observed and the development of GC-like structures is detected in a few mice. The fact that not all mice display thymic GCs could also reflect what is observed in the human disease as not all patients have thymic

GCs. Altogether, this EAMG model recapitulated better the human pathology than the classical model with C57BL/6 mice which do not show thymic pathology. Moreover, data suggest that thymic follicular hyperplasia is the result of combined features, including overexpression of CXCL13 and increased inflammation due to CFA [40].

This novel transgenic mouse model is of particular interest as it finally mimics the thymic pathology observed in human MG.

### Conclusion

Abnormal TLR signaling activation and uncontrolled resolution of inflammation are suspected to play a key role in the development of autoimmune diseases and possibly in the chronicity of these diseases. Experimental models have been developed for numerous autoimmune diseases. These models almost all rely on animal immunization with an antigen solubilized in CFA (Table 2). It is clear that CFA effects are mainly due to MTB and the activation of TLR signaling pathways. Diverse attempts in research laboratories have been made to try to substitute CFA by the use of TLR agonists, such as LPS and CpG known to activate TLR4 and TLR9, respectively, as MTB does. LPS with IFA have proved to be efficient to induce EAMG and thyroiditis [101, 103]. The results obtained with CpG-containing oligodeoxynucleotides are more contradictory as TLR9 activation seems to protect animal in experimental autoimmune models, such as diabetes, Sjogren's syndrome, and encephalomyelitis models [123, 124, 125].

If dsRNA signalization is not the main mode of action of microbial patterns displayed by MTB, poly(I:C) has nevertheless been largely tested and proves to be efficient with or without CFA to induce experimental autoimmune models [107, 108, 109, 110, 111, 112].

As the use of CFA is more and more restricted because of painful adverse effects and the restrictions of national regulatory authorities, the development of alternative adjuvants for induced experimental autoimmune models is a necessity for the incoming years. However, the most difficult challenge will be to switch to these new models as the literature on induced models using CFA is rich. These models have been used for years to better understand pathological mechanisms and to test new therapeutic approaches. For experimental models based on the use of TLR agonists, we do not have yet so much hindsight. However, an ideal alternative adjuvant resides probably in the use of a mix of different TLR agonists.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors have declared that no conflict of interest exists.

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