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Mélanie Cron

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Sorbonne Université

Ecole doctorale 394 : Physiologie, physiopathologie et thérapeutique

Sorbonne Université - INSERM - AIM - Centre de Recherche en Myologie

Implication of microRNAs in the pathophysiology of autoimmune Myasthenia Gravis

Implication des microARN dans la physiopathologie de la Myasthénie Autoimmune

Par Mélanie CRON

Thèse de doctorat de Biologie

Dirigée par le Dr Rozen Le Panse

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ABBREVIATIONS

3'UTR	3' untranslated region
ACh	Acetylcholine
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
ADAR	Adenosine deaminase acting on RNA
AID	Autoimmune disease
AKT	Serine-Threonine Protein Kinase
ASO	Antisense oligonucleotide
BCR	B-cell receptor
bp	Base pair
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CCR4-NOT	Carbon catabolite repressor 4-NOT
CD	Cluster of differentiation
CDS	Coding DNA sequence
CFA	Complete Freund's adjuvant
ciRs-7	Circular RNA sponge for miR-7
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell
DGCR8	DiGeorge critical region 8
DN	Double negative
DP	Double positive
dsRNA	Double-strand RNA
EAMG	Experimental autoimmune myasthenia gravis
EBV	Epstein-Barr virus
eIF4	Eukaryotic initiation factor 4
EOMG	Early-onset myasthenia gravis
EpCAM	Epithelial cell adhesion molecule

FOXP3	Forkhead box 3
GC	Germinal center
GWAS	Genome wide association study
HDAC	Histone deacetylase
HET	Heterozygous
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IFNAR	IFN-I receptor
IFN-I/- α /- β /- γ	Type-I interferon / interferon alpha / beta / gamma
IVIg	Intravenous immunoglobulin
KO	Knock-out
LEV	Lymphatic endothelial venule
LNA	Locked nucleic acid
LOMG	Late-onset myasthenia gravis
LPS	Lipopolysaccharide
LRP4	Low density lipoprotein receptor-related protein 4
MAPK	Mitogen-activated protein kinase
MG	Myasthenia gravis
MGFA	Myasthenia gravis foundation of america
MHC	Major histocompatibility complex
MIR	Main immunogenic region
miRNA	MicroRNA
mRNA	Messenger RNA
MuSK	Muscle specific kinase
NMJ	Neuromuscular junction
NSG	NOD scid gamma
nt	Nucleotide
PABP	Poly(A) binding protein
PAN2/3	Poly(A) specific ribonuclease subunit 2/3
pDC	Plasmacytoid dendritic cell
Poly(I:C)	Polyinosinic–polycytidylic acid

Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RISC	RNA-induced silencing complex
RLCV	Rhesus lymphocryptovirus
RNAi	RNA interference
siRNA	Silencing RNA
SLO	Secondary lymphoid organ
SNP	Single nucleotide polymorphism
ssRNA	Single-strand RNA
TCR	T-cell receptor
TEC	Thymic epithelial cell
TGF- β	Transforming growth factor beta
TK	Thymidine kinase
TLO	Tertiary lymphoid organ
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFAIP3	TNF alpha induced protein 3
TNFRSF11A	TNF receptor superfamily member 11a
TNIP1	TNF alpha induced protein 3 interacting protein 1
TRBP	Trans-Activation Responsive RNA-Binding Protein
TSA	Tissue specific antigen
TUT	Terminal uridylyltransferase
WT	Wild-type
XPO5	Exportin 5
XRN1	5'-3' exoribonuclease 1

LIST OF PUBLICATIONS

Cron, M.A., Maillard, S., Robinet, M., Fadel, E., Guihaire, J., Liston, A., Berrih-Aknin, S. and Le Panse, R. "Involvement of miR-29s in the pathogenesis of autoimmune Myasthenia Gravis." **in preparation**

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"Causes and consequences of miR-150-5p dysregulation in Myasthenia Gravis." **submitted**

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"Analysis of microRNA expression in the thymus of Myasthenia Gravis patients opens new research avenues." *Autoimmun Rev* 17(6): 588-600.

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"Use of Toll-Like Receptor Agonists to Induce Ectopic Lymphoid Structures in Myasthenia Gravis Mouse Models." *Front Immunol* 8(1029).

Robinet, M., Villeret, B., Maillard, S., **Cron, M.A.**, Berrih-Aknin, S. and Le Panse, R. (2016).

"Review on Toll-Like Receptor Activation in Myasthenia Gravis: Application to the Development of New Experimental Models." *Clin Rev Allergy Immunol.* 52(1): 133-147. **Review**

I. STATE OF THE ART

This thesis aims at evaluating and characterizing the implication of various microRNAs (miRNAs) in the context of an autoimmune disorder, Myasthenia Gravis (MG). miRNAs are small molecules of non-coding RNA, characterized as epigenetic factors thanks to their ability to interact with messenger RNA (mRNA). miRNAs are being investigated in many diseases, including autoimmune diseases (AIDs). AIDs' etiology is still largely unknown and unfortunately, MG is not an exception. In this context, I investigated the etiological and pathophysiological implications of miRNAs in MG with different approaches.

1. miRNAs: what are they, how do they work and for what purpose?

1.1. Discovery and first characterization of RNA interference

In 1984 Izant and Weintraub conducted the first experiment, aimed at inhibiting the expression of a gene with the formation of an RNA duplex [1]. The authors used plasmid constructs coding for the thymidine kinase (TK) to be expressed in TK-deleted mouse cell lines (TK⁻ L cells). In parallel, they used a recombinant DNA, coding for the antisense RNA of the TK gene, so that the cells could express antisense RNA by themselves. With this design, the authors could evaluate in which proportion the RNA antisense was able to downregulate TK expression, without background noise. The authors saw a decrease in the TK activity in cells injected with antisense RNA construct. However, they could not determine the efficiency of the inhibition, the stability of the antisense RNA or the localization of the inhibition.

In the late 1980's, A.R. Suitje's team described changes in the pigmentation pattern of petunia and tobacco flowers after the introduction of an "antisense chalcone synthase gene", a gene coding for a key enzyme of the flavonoid biosynthesis pathway that is involved in the pigmentation of the flowers [2]. The authors suggested that these changes were due to the presence of RNA duplexes in the flowers tissue. They also highlighted that differences observed in some patterns were the result of different integration positions of the antisense chalcone synthase gene, pointing out the importance of the transgene positioning. Later on, R. Jorgensen's team came to the same conclusions [3].

In 1991, A. Fire's team managed to inhibit gene expression in the muscle of the worm *Caenorhabditis elegans* by injecting plasmids coding for antisense RNA of myofilament proteins in *C. elegans* oocytes. These injections resulted in disorganization of the myofilaments and thus slow movements of the animals. Good integration of the antisense RNA allowed the authors to see these defects in the next generations [4]. Still in *C. elegans*, the temporal down-regulation of the *lin-14* gene by *lin-4* was explained in wild-type (WT) worms [5, 6]. These studies showed for the first time that a RNA duplex could result in a lower protein expression *in vivo*.

The term "RNA interference" (RNAi) was used for the first time by A. Fire and C. Mello in 1998, in a study where they prove that the injection of double-strand RNA (dsRNA) is more efficient than the injection of a single-strand RNA (ssRNA) to induce the inhibition of endogenous myofilament proteins, compared to other previous studies, including their previous one [4, 7]. This study was the beginning of many investigations about RNAi. A. Fire and C. Mello received the Nobel Prize in Physiology or Medicine in 2006 for their discoveries.

1.2. Characterization of miRNAs

Among RNAi, two main types of different RNA can be distinguished: small interfering RNAs (siRNAs) and microRNAs (miRNAs). siRNAs are exogenous ssRNAs, coming from transposons movements, infections or transgene activity [8]. miRNAs are endogenous dsRNA processed into ssRNAs and involved in the regulation of physiological phenomena [9]. There are several miRNA-biogenesis pathways: the canonical pathway (the most common pathway) and alternative pathways for specific miRNAs and miRNA families [10]. It is noteworthy that long non-coding RNAs (around 200-nt long) are not considered as RNAi as they do not directly act on mRNA but act epigenetically [11].

1.2.1. Nomenclature

miRNAs are named by the prefix "miR" and a single identifying number (miR-150, miR-7...) along with its coding gene, according to the gene nomenclature of the organism (*MIR150*, *MIR7* for human per example). Ortholog miRNAs are named identically among species. If needed, three letters referring to

the species can be placed before the prefix: “*hsa*” for *Homo sapiens*, “*mmu*” for *Mouse musculus*, “*cel*” for *C. elegans*...

If two miRNAs share a common sequence but are transcribed from different loci and have distinct precursors, a number is placed at the end (miR-7-1, miR-7-2...). Similarly, if two miRNA sequences differ only by one or two nucleotides (nt), they will be noted miR-29a, miR-29b, miR-29c... These rules can be added if the conditions require, such as the miR-29 family: miR-29a, miR-29b-1, miR-29b-2, miR-29c. Finally, orientations are very often noted at the end of the name: miR-150-5p, miR-150-3p. If the guide and the passenger strand can be differentiated among the two strands, the passenger strand will be noted with a star (*). It is noteworthy that the passenger strand can either be the -5p or the -3p strand [12, 13].

An exception is made for the lethal-7 (*let-7*) family. *Let-7* was first discovered in *C. elegans* as a time controller for the development of the worm. Later on, orthologs were found in humans and other species but the denomination of the miRNA was not changed. In humans, the *let-7* family is composed of 10 mature miRNAs [14].

1.2.2. miRNA biogenesis pathways

Canonical miRNA-biogenesis pathway

As shown in Figure 1, miRNAs are transcribed from specific miRNA genes by the RNA polymerase II. The resulting transcript is a primary miRNA (pri-miRNA) characterized by a hairpin, a very specific form adopted by miRNAs during their biogenesis. The pri-miRNA can reach 1 kilobase (kb) length and is composed of a 33-35 base pair (bp) stem ending by a loop and two ssRNA with 5' and 3' end flanking the stem [15]. Next, the pri-miRNA is cleaved by DROSHA, a RNase III, and its partner, DiGeorge Critical Region 8 (DGCR8), together called the microprocessor. The association of these nuclear proteins is called the microprocessor, with a single DROSHA molecule for two molecules of DGCR8. Here, the role of DROSHA is to set the cleaving point on the pri-miRNA, around 11bp from the basal junction and 22 bp from the apical loop. DGCR8 stabilizes DROSHA and enhances precision and efficiency of the cleavage [16].

After cleavage, the 60nt-length precursor miRNA (pre-miRNA) is exported from the nucleus to the cytoplasm by the protein exportin 5 (XPO5). XPO5, which is associated with a Ran-guanosine triphosphate (Ran-GTP) cofactor to enhance the export, binds to the pre-miRNA in the nucleus and releases it in the cytoplasm, along with the hydrolysis of the Ran-GTP, becoming Ran-GDP [17, 18]. Pre-miRNAs have the particularity to have a 2 nt 3' overhang that allows the recognition of the RNase III DICER in the cytoplasm. DICER, thanks to its PAZ domain allowing it to identify ssRNA, will recognize the pre-miRNA and will cut out the loop, resulting in a miRNA duplex of 20-23 nt. DICER's partner, the Trans-Activation Responsive RNA-Binding Protein (TRBP), has a similar role with DICER that DGCR8 has with DROSHA, which is helping DICER to stabilize the cleavage of the pri-miRNA by increasing its half-life [19].

Subsequently, the miRNA duplex is loaded into the RNA-Induced Silencing Complex (RISC). The RISC is a two-step transient association involving DICER, TRBP, AGO proteins and heat-shock proteins acting as chaperones, whose role is to select the leading strand to induce mRNA silencing. In mammals, we have four distinct AGO proteins (1-4) but only AGO2 displays a catalytic activity. First, the miRNA duplex is loaded from DICER onto AGO2, which is associated with two chaperones, HSC70 and HSP90, allowing it to adapt its conformation to the duplex. Next, the duplex is unhybridized by AGO2, leaving the non-chosen strand, called the passenger strand, out of the RISC to be discarded. Once the leading strand, in its "final" miRNA form, is loaded into AGO2, the chaperones leave the complex [20, 21]. The choice of the leading/passenger strand is not arbitrary. The strand with the lower 5' thermodynamic stability will preferentially be the leading (or guide) strand whereas the strand displaying a higher 5' stability will be the passenger strand [8, 22].

The ultimate goal of miRNAs is to prevent the expression of target proteins by acting on mRNA. According to the perfection of the sequence matching between a miRNA and its target mRNA, the mechanism of inhibition of protein expression will not be the same. In plants, miRNA-mRNA pairing is totally or nearly perfect. Consequently, the RISC proteins, AGO proteins in particular, function as endonucleases, slicing the mRNA complementary to the guide strand [23]. In mammals, the base

pairing is almost never perfect on the whole miRNA length. However, as detailed by Filipowicz *et al.*, some rules “need” to be respected in order to have a correct translational repression: the pairing must be perfect between the nt 2 to 8 of the miRNA (called the “seed region”) and the 3’ end of the mRNA (3’UnTranslated Region or 3’UTR), pairing between the 3’ end of the miRNA and the 5’ end of the mRNA should be sufficient enough to stabilize the reaction and mismatches should be present to prevent any cleavage from AGO2 [24].

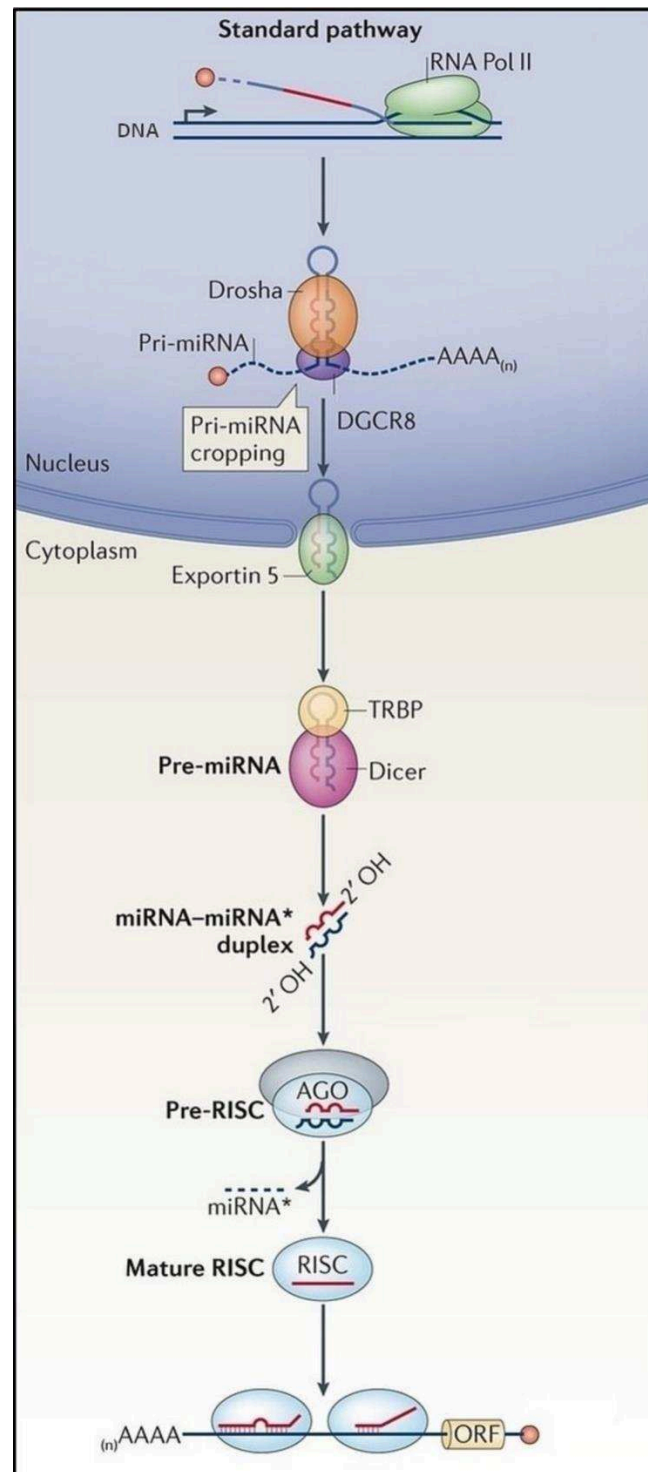


Figure 1: Canonical biogenesis pathway of miRNAs

miRNAs are transcribed from specific genes by the RNA Polymerase II, a 12-subunit enzyme involved in the transcription of mRNAs. When transcribed, the transcript, called pri-miRNA, adopts a particular conformation in a hairpin. DROSHA and DGCR8 will isolate the hairpin from the rest of the transcript and XPO5 will mediate the transport from the nucleus to the cytoplasm. Then, DICER and TRBP will cut the apical loop of the pri-miRNA, now becoming pre-miRNA. The pre-miRNA is a duplex of miRNA, and according to 5'-end thermodynamic stability, only one strand (the leading strand, without *) will be loaded in the RISC, composed, among other proteins, of AGO2. AGO2 is the only member of the family displaying catalytic activity, allowing it to dissociate the miRNA duplex. The RISC will lead the miRNA to its mRNA target and induce translational repression or in rare cases, mRNA cleavage. Adapted from [25].

DROSHA/DGCR8-independent biogenesis pathway

Also called the “miRtron” pathway, this alternative biogenesis pathway was the first to be described by Bartel and Lei’s teams in 2007 [26, 27]. As shown in Figure 2A, miRtrons are derived from spliced introns with a strong hairpin formation potential. Briefly, splicing is catalyzed by the spliceosome, composed of 5 ribonucleoproteins (U1, 2, 4, 5 and 6) associated with other proteins. Splicing consists in two major steps: 5’ cleavage and lariat formation of the intron and 3’ cleavage and exon association [28]. Then, the lariat takes a “canonical” pre-miRNA form after the intervention of the lariat debranching enzyme DBR1 [29, 30]. Afterwards, the pre-miRNA can follow a classical biogenesis as detailed above.

DICER-independent biogenesis pathway

So far, this biogenesis pathway has been described only for miR-451, a highly conserved miRNA among vertebrates [31-33]. As shown in Figure 2B, miR-451 is processed like any other miRNA following the canonical pathway. After the first cleavage by DROSHA/DGCR8 and the export to the cytoplasm, the pre-miR-451 is too short to be processed by DICER and TRBP (around 20 nt long). Pre-miR-451 is loaded onto AGO2, which will exert its slicing activity to isolate the leading strand, helped by the exonuclease PARN [34].

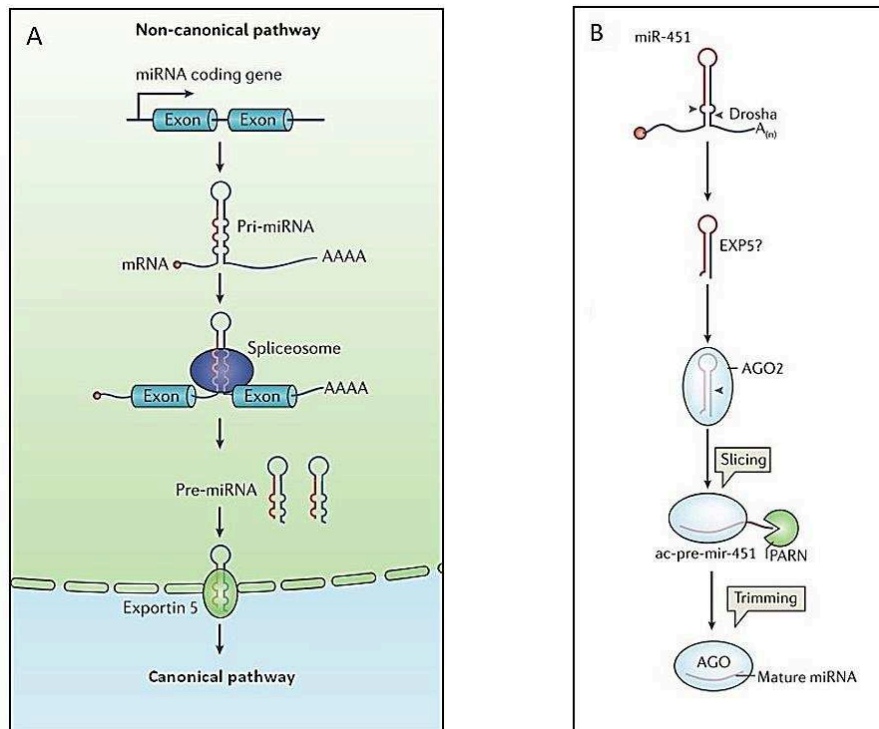


Figure 2: Non-canonical biogenesis pathways: miRtrons and DICER/TRBP-independent pathway
 (A) Some miRNAs are transcribed from introns. In this case, these miRNAs result from a spliced intron, isolated from flanked exons by the spliceosome. Once under the « classical » pre-miRNA form, they bypass the DROSHA/DGCR8 step and are exported by XPO5 and are able to follow the canonical biogenesis pathway. (B) For now, only miR-451 was described as bypassing the DICER/TRBP step for its biogenesis. It is transcribed and processed by DROSHA and DGCR8 and then exported to the cytoplasm. miR-451 is not long enough to be processed by DICER and TRBP (17 nt stem and 4 nt loop) and therefore, is loaded in AGO2, which will isolate the mature form of miR-451 with the help of the exonuclease PARN. Adapted from [15, 35].

DROSHA/DGCR8 and DICER-independent biogenesis pathway

Called “agotrons”, these “regulatory” small RNAs are derived from short introns and are processed directly through AGO2, bypassing the DROSHA/DGCR8 and DICER/TRBP steps. Very recently described, these RNAs act similarly to miRNAs by binding to targets with seed complementarity [36]. However, functions of such RNAs need to be further investigated. Hansen’s group suggests that agotrons could serve as a guide to avoid off-target effects or be useful as stabilizers of Ago proteins [10].

1.2.3. Regulation of miRNA biogenesis

miRNA biogenesis can be regulated at each step detailed above. As the canonical biogenesis pathway is the most common, I will focus on the regulation of the elements of this particular pathway.

1.2.3.1 Regulation of miRNA gene transcription

miRNAs are transcribed from specific miRNA genes. These genes can be transcribed independently (intergenic miRNAs) or inside the intron of a host gene (intronic miRNAs), supposedly under the control of the host gene promoter [37, 38]. However, 35% of intronic miRNAs might have their own regulatory elements functioning as promoters [39]. The identification of such promoters is difficult but can be facilitated by the detection of CpG islands, transcription start sites and conserved transcription factors binding sites. These elements imply that epigenetic modifications can happen in this particular region, suggesting the presence of promoters.

Methylation is a broadly spread mechanism to regulate gene expression but also miRNA expression. It adapts the chromatin conformation to induce or to repress the transcription of a region by adding or removing methyl groups thanks to DNA methyltransferases (such as DNMT1 or 3). So far, methylation of miRNA promoters was mainly described as one of the causes of several cancers [40], but this mechanism is most likely applied in physiological conditions too.

miRNA transcription can also be regulated via histone deacetylases (HDACs). HDACs act as modifiers of the chromatin conformation, along with histone acetylases, to facilitate or prevent the transcription. Some transcription factors also act on the transcription of particular miRNAs such as MYC, P53 or the MYOD1, among others [15, 41].

1.2.3.2 Regulation of the miRNA microprocessor

It is very important that DROSHA and DGCR8 remain stable in the context of the canonical biogenesis pathway. They are involved in a loop where they stabilize and interact with each other, even when they are not involved in the cleavage of the pri-miRNA [42]. Both DROSHA and DGCR8 also undergo post-transcriptional modifications. For example, DROSHA is phosphorylated by the Glycogen Synthase Kinase 3 β (GSK3- β) to ensure its nuclear localization [43, 44], just as DGCR8 is phosphorylated by the extracellular signal-regulated kinases (ERK) 1/2, in order to stabilize it [45]. DGCR8 is at the heart of a tight balance of acetylation where deacetylation by HDAC increases the affinity for pri-miRNAs and acetylation prevents degradation [46]. This particular and rapid form of regulation is useful if the

stoichiometry of the microprocessor is affected. Indeed, if DGCR8 is expressed three times more than DROSHA, this will result in a decreased processing activity of DROSHA [47].

The regulation of the export following the DROSHA/DGCR8 processing was less investigated and it will be interesting to have a look at it, as well as more generally at the regulation of Ran-GTP proteins.

1.2.3.3 Regulation of the DICER/TRBP complex

TRBP, as the major partner of DICER, is tightly regulated, so that DICER can assume its functions. It is phosphorylated by MAPK (ERK 1/2) to be stabilized. It is noteworthy that TRBP and DICER stability is increased when they are bound together [45]. DICER can be affected and regulated by exogenous signals, such as infections (mimicked by polyinosinic-polycytidylic acid (Poly(I:C)) and type-I interferon (IFN-I)), but also cellular stress (such as reactive oxygen species) [48]. Ha & Kim comprehensively reviewed a majority of DICER regulators especially proteins binding to the pre-miRNA at the DICER/TRBP step [15]. It is interesting that the adenosine deaminase acting on RNA family (ADAR, composed of ADAR1 and ADAR2) promotes gene editing and is complexed with DICER [49]. ADAR proteins catalyze a reaction that turns adenosine into inosine, read as guanosine. This results in a destabilization of the binding (a A:G association is less stable than a A:T association) leading to an increased susceptibility of a dsRNA to become single-stranded [50]. In the context of ADAR1/DICER association (ratio 1:1), ADAR1 helps to generate more miRNAs by increasing DICER efficiency, as TRBP [49].

1.2.3.4 Regulation of Argonaute proteins and accessibility to target mRNAs

AGO2 is reported to be stabilized by hydroxylation by the type I collagen prolyl-4-hydroxylase and its localization in the cytoplasm and in particular in P-bodies is ensured by its phosphorylation by AKT3 [51, 52]. Moreover, the epidermal growth factor receptor (EGFR) phosphorylates AGO2 in response to hypoxia. This phosphorylation results in the dissociation of AGO2 from DICER to diminish the production of miRNAs [53]. Another modification recently described is the regulation via Poly(ADP-ribose) polymerases. These enzymes will affect the accessibility of the AGO2/miRNA complex to the target mRNA by acting on electrostatic charges, especially upon viral and bacterial infections [54, 55].

1.3. Modes of action and biological roles of miRNAs

miRNAs are highly conserved among vertebrates, flies and worms. In mammals, they represent about 2% of the genome but can possibly regulate 60% of protein-coding genes [56]. The following part will describe how miRNAs interact with their mRNA targets and to what extent they act on protein output and other biological roles.

1.3.1. Target recognition and mRNA-miRNA interactions

1.3.1.1 Basic principles

miRNAs are known to bind to mRNA in their 3'UTR (5' end of the miRNA) [57]. However, studies show that 5'UTR is just as efficient as 3'UTR to induce miRNA-mediated gene silencing on mRNAs, hence, to be more accurate, authors introduced the term “miRNA responsive element”, as a binding site of miRNAs on mRNAs [58]. Some studies also showed transcripts with miRNA binding sites in their open reading frames but thought they were too rare to be noted [59]. As a result, the different databases listed below allow researchers to look for miRNA responsive elements in 3'UTR, in coding DNA sequence (CDS) or in genomic sequences.

As mentioned in paragraph 1.2.2, miRNA-mRNA interaction relies on the perfect matching of the entire sequence between the nt 2 and 7 of the miRNA, called the seed region. Actually, four types of seed sites on mRNA are listed, as shown in Figure 3:

- the 6mer, the seed region itself,
- the 7mer-m8 site, consisting of the seed region and an additional match at nt 8,
- the 7mer-A1 site, consisting of the seed region and an adenosine in position 1,
- the 8mer, consisting of a mix of the two previous sites, *i.e.* the seed match, an additional match in position 8 of the miRNA, and an adenosine in position 1.

The recognition of one of these sites may be sufficient to induce an effect on the mRNA target, but the additional pairing on the mRNA seems to enhance mRNA recognition by miRNAs. The extensive pairing of the nt 12 to 17 of the miRNA and in particular of nt 13 to 16 could balance imperfect matching in

the seed region [60]. It is worth noting that, the 6mer seed regions display a lower efficiency in targeting mRNA than 7mer-m8 [56]. The seed region was considered a major condition for the matching of miRNA on the mRNA because it is conserved among organisms [61], the majority of mRNA were under selective pressure and evolved to maintain the pairing with miRNA thanks to the seed region [62] and mutations or bulges of a single nt in the seed (typically G:U base pair) resulted in a less efficient targeting [57].

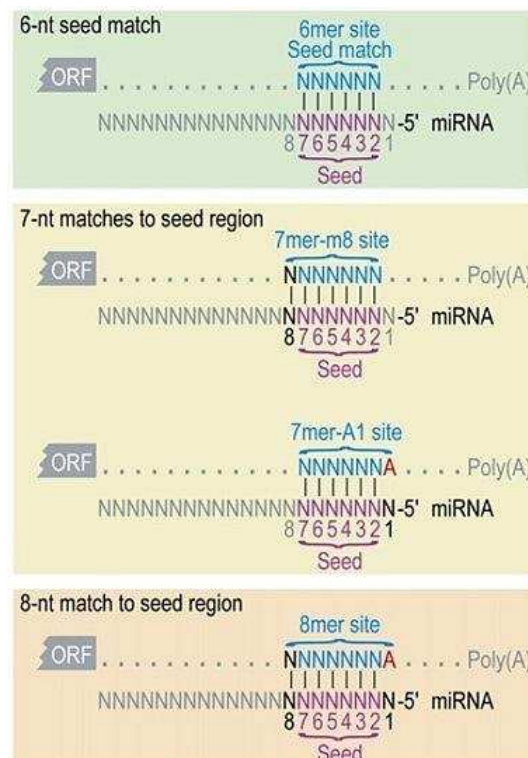


Figure 3: miRNA-mRNA interactions

In mammals, the perfect matching between miRNA and targeted mRNA almost never occurs. However, to induce miRNA-mediated silencing, a perfect matching must be done between the “seed” region at the 5’ end of the miRNA and the mRNA. Strictly speaking, the “seed” region represents the six nucleotides at position 2 to 7 of the miRNA (green frame). Specificity of the matching can be increased if the “seed” is enlarged by one or two nucleotides. Two conformations involving 7 nucleotides and one conformation involving 8 nucleotides are found. Adapted from [60].

1.3.1.2 Prediction of target sites and diversity of databases

After the characterization of miRNAs and their interaction with mRNA, an important point is to know which miRNA can target which mRNA, and inversely. It is interesting that a single miRNA can have several mRNA targets and mRNAs display several binding sites for different miRNAs [63]. To make those data more easily available, research teams created different databases. Here, I will describe

three databases/algorithms that I used during my PhD among the existing ones, comprehensively reviewed in [56, 64]:

- TargetScan was the first to be created in the field by DB. Bartel and CB. Burge's group [61]. TargetScan is a tool allowing users to find miRNAs matching with the 3'UTR of mRNAs using the seed region; it extends the pairing to additional nt (as detailed above) and assigns a rank to 3'UTR according to several thermodynamic parameters. TargetScan also takes the conservation of the binding site on the mRNA among several species into account [62].
- One of the most used algorithms is miRanda [65], available through the "microrna.org" website [66]. The algorithm is based on the matching between miRNAs and the 3'UTR of the mRNA. miRanda has the advantage of displaying a score evaluating the impact of the miRNA on the mRNA (mirSVR score); furthermore, it does not limit matching searches to 3'UTR but also includes genomic sequences [64].
- The DIANA-microT database, which is specialized for searching matching CDS and 3'UTR, also indicates the recognition between a miRNA and targets containing a single miRNA responsive element [67]. DIANA-microT also takes the G:U mismatches and the proteins associated with the miRNAs (from the RISC) into account, whose conformation could interfere with the theoretical matching [64].

1.3.2. Mechanisms of post-transcriptional regulation of mRNA by miRNAs

miRNAs' main mode of action to prevent the expression of specific proteins is post-transcriptional regulation. As detailed previously, when the matching between the miRNA and its target is perfect, mRNA will be cleaved immediately. This phenomenon, which is very common in plants, almost never happens in mammals. After their fixation on mRNA, miRNAs induce several mechanisms including mRNA destabilization and repression of translation. It is not quite clear how miRNAs directly induce these mechanisms but some evidence was found. The main mechanisms are illustrated in Figure 4.

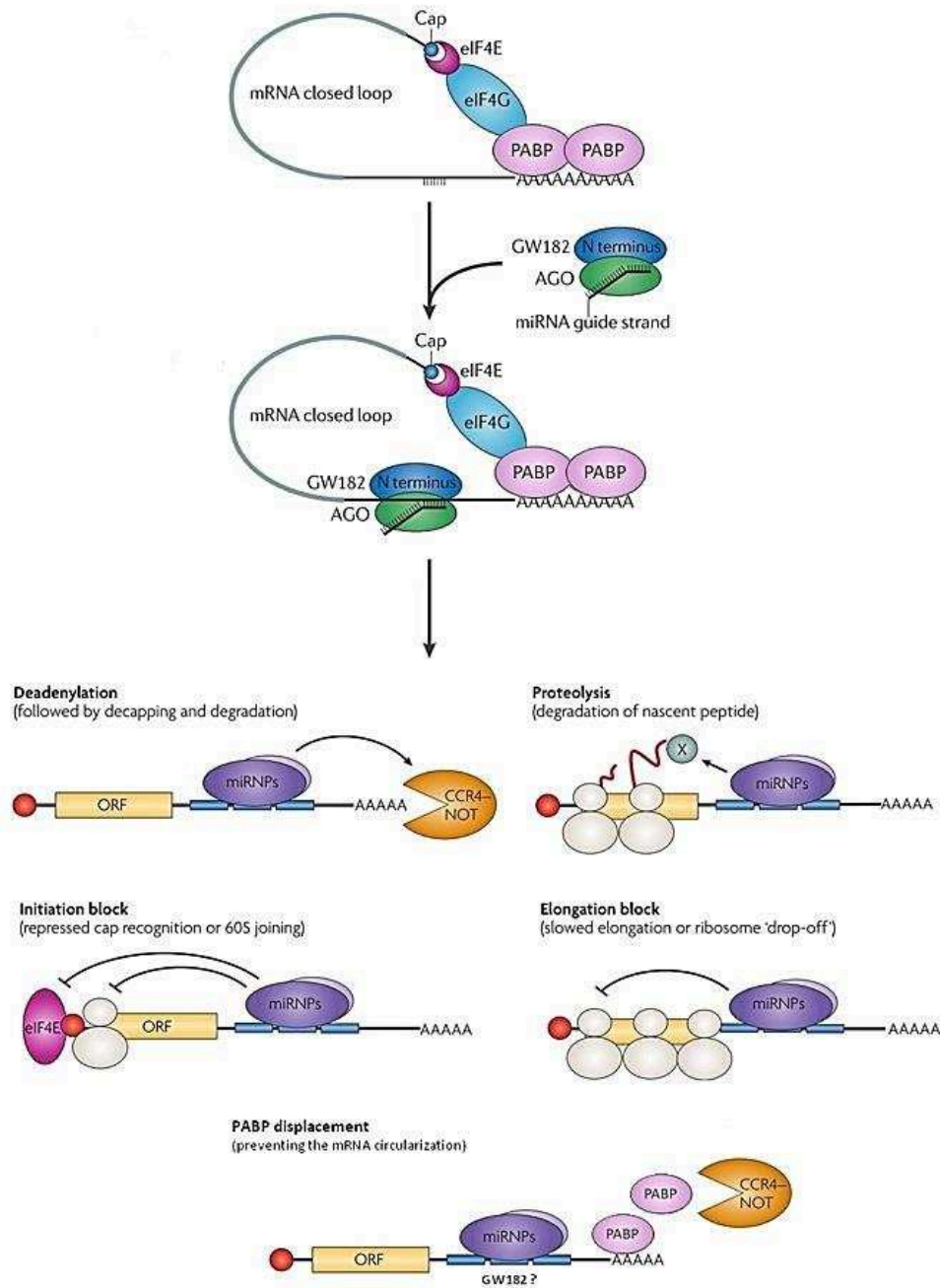


Figure 4: Possible mechanisms of miRNA-mediated repression

Initiation of translation begins with the circularization of mRNA. This binding will induce several possible mechanisms to repress the translation. mRNAs can be deadenylated by the recruitment of the CCR4-NOT complex by GW182 or can induce the blockade of the recognition of the 5' cap by eIF4E (left). After initiation of translation, miRNAs might be able to induce ribosome dissociation, the degradation of the newly produced peptide (right) or PABP displacement (middle). Adapted from [24, 68].

1.3.2.1 Deadenylation and decay of miRNA targets

Destabilization and deadenylation of the miRNA targets are the most widespread mRNA decay mechanism in mammals. The mechanisms of mRNA decay after miRNA fixation are reviewed by Jonas and Izaurralde [69]. To summarize, after the fixation of the miRNA on its mRNA target, AGO2 recruits members of the P-body, or GW-body proteins. P-bodies are small granules localized in the cytoplasm and enriched in proteins involved in the mRNA metabolism, including deadenylation and decay [24, 70]. These adaptor proteins then recruit the Poly(A) specialized Nuclease 2 & 3 complex (PAN2-PAN3), proteins specialized in the deadenylation of mRNA (whether it is miRNA-mediated or not) by binding to the poly(A) tail. GW182, a protein of the P-bodies, also recruits the Poly(A) Binding Protein (PABP), which will be fixed on the poly(A) tail and will further induce the circularization of the mRNA and the mRNA decapping. PAN2, thanks to its catalytic activity, is able to rapidly induce the shortening of the poly(A) tail. Nonetheless, the PAN2-PAN3 complex is known to be part of the initial phase of deadenylation and sometimes, for reasons that are not clear, it will not complete the full degradation. The second phase of the deadenylation process is fulfilled by the CCR4-NOT complex, another complex recruited by GW182. Composed of nine subunits, including two catalytic subunits, these proteins are known to form the main mRNA-deadenylation complex. The CCR4-NOT complex shares several characteristics with the PAN2-PAN3 complex: their localization in the cytoplasm, their catalytic activity, and being components of P-bodies. The functions of the two complexes can be redundant but compensate for loss-of-function mutations [71]. These redundancies can be explained by the need to prevent any absence during processes, as the proteins involved in the mRNA metabolism and decay play an essential role.

Once deadenylated, mRNAs are uridylated by the enzymes TUT4 and TUT7 at the 3' end. This uridylation is considered as a mark for downstream proteins to pursue the decay [72]. Next, mRNAs undergo decapping at the 5' to expose them to exonucleases. This step is performed by the enzyme Decapping Protein 2 (DCP2) and one of its many cofactors, Decapping Protein 1 (DCP1) [73]. In the end, the enzyme 5'-to-3' exoribonuclease (XRN1) will finalize the mRNA decay by totally degrading it [69].

1.3.2.2 Inhibition of the translation of the miRNA target

In addition to the mRNA deadenylation and decay, miRNAs mediate silencing through translation initiation inhibition according to four distinct mechanisms: PABP movement mediated by GW182, recruitment of translational repressors, non-recognition of the cap by eIF4E and ribosome drop-off [74].

- Izaurralde's team showed that PABP is dissociated from translation initiation factors by the CCR4-NOT complex recruited by GW182, resulting in the non-circularization of the mRNA. As this conformation being compulsory for the degradation of mRNA, the mRNA is not degraded but its translation is impaired [75].
- Independently of mRNA deadenylation, deadenylases are also able to induce translation repression. Wickens' group demonstrated that CAF1, a protein of the CCR4-NOT complex, could induce the repression of translation by itself, whether the mRNA is deadenylated or not [76]. CAF1 and CCR4 are also able to suppress poly(A) residues upstream of the poly(A) tail, possibly previously deadenylated. This observation suggests that these enzymes play another role in miRNA-mediated translational repression [77].
- When translation is initiated, eIF4E is associated with eIF4G to induce mRNA circularization by binding to PABP [68]. T. Preiss' group demonstrated that miRNA-mediated repression of translation could happen during initiation by targeting eIF4E, whether by inducing the non-binding to the 5' cap of the mRNA or blocking its function when bound to the cap [78].
- Several miRNAs were shown to act at the post-initiation stage of the translation to induce the ribosome drop-off. At the translation initiation, the 40S and 60S ribosomal subunits assemble on the mRNA to form polysomes (or polyribosomes), leading to translation and protein production. PA. Sharp's group demonstrated that miRNAs could prematurely induce the dissociation between ribosomes and targeted mRNA [79], which was contradicted by studies led by JD. Richter and TW. Nilsen. They observed that ribosomes were still "attached" to mRNA and hypothesized that miRNAs prevent the association of polypeptides from ribosomes

[80, 81]. Nonetheless, Iwakawa and Tomari confirmed the theory of Petersen and colleagues *in vitro* in plants [82]. In the meantime, Tamori's team interestingly demonstrated that GW182 could block the 80S ribosomal complex association in *Drosophila (D.) Melanogaster* by the upstream dissociation of eIF4A, a RNA helicase [83, 84].

However, the functional mechanisms underlying the translational repression are very unclear. The evidence detailed above provides clues to decipher the different translational repression mechanisms, but to my knowledge, no clear pathways have been described.

1.3.3. Biological roles of miRNAs

1.3.3.1 miRNAs as regulators of developmental processes

miRNAs often act to reinforce cellular destinies by acting on developmental and/or homeostasis pathways. It is the case in *C. elegans* during neurogenesis, where miR-124 is expressed during the transition of neuro-ectodermal progenitors to mature neuronal tissue. In the future neuronal tissue, miR-124 is highly expressed, resulting in a low expression of its target mRNAs. However, in epidermal cells, derived from the same progenitors and that do not express miR-124, miR-124 targets are increasingly expressed compared to the neuronal tissue. With such a process, "epithelial" genes targeted by miR-124 are repressed in the neuronal tissue, ensuring that the differentiation occurs correctly [85].

miRNAs act following the coherent feedforward loop (Figure 5A). This motif is a typical model where the expression of the target mRNA is inversely correlated with the expression of a miRNA and the most widespread model recapitulating miRNA-mediated regulation. For example, element A (a given miRNA-inducer gene) activates element B (a miRNA) and both inhibit element C (target gene). Element B, induced by element A, accumulates and is able to compensate any disturbance in element A production by acting on element C with the same effect [9].

1.3.3.2 miRNAs acting as noise reducers in gene expression

As raised by Schmiedel *et al*, how do miRNAs, which weakly repress a vast number of targets and whose knockdown often does not result in a pronounced phenotype [86, 87], remain conserved among species? The hypothesis is that miRNA regulation of gene expression is very tight and precise and that miRNAs are able to reduce background noises [88]. Elowitz *et al.* described two types of noises: intrinsic and extrinsic. Intrinsic noise corresponds to biochemical processes during gene expression whereas extrinsic noise reflects events happening in other cellular compartments [89]. Overall, noise arises from random events such as cell-to-cell expression variation and fluctuations over time of protein degradation, translation etc...[90]. To what extent can miRNAs act on noise reduction?

For intrinsic noise, an important parameter to take into account is the transcription and translation rates that will influence protein levels. If the transcription rate is low (a few mRNA transcribed), the translation rate will be more important to maintain a certain pool of proteins. Knowing that translational repression represents 16% of miRNA-mediated repression [91], miRNAs will preferentially tend to reduce intrinsic noises by acting on the mRNA destabilization and decay.

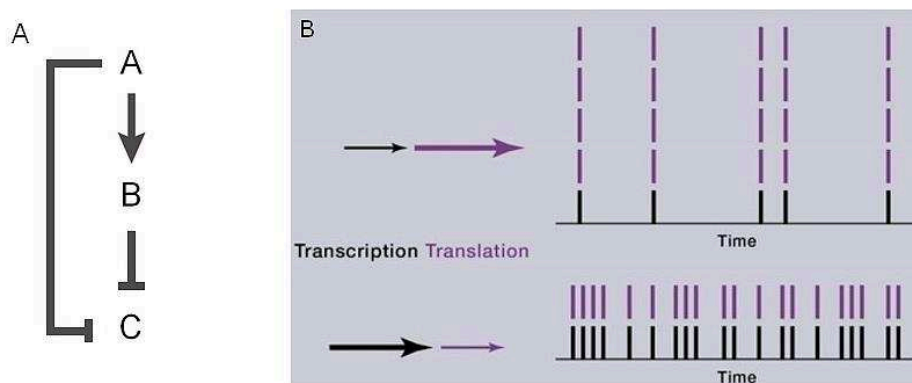


Figure 5: Functions of miRNAs: robustness during development and noise reduction

(A) The coherent feed forward loop represents the typical case where miRNA and its target's expressions are inversely correlated. This way, action on element C is reinforced and changes in the expression of element A or B can be compensated. (B) miRNAs act as noise reducers and act preferentially during transcription to induce mRNA destabilization and decay, rather than inducing translation repression. Adapted from [9, 92].

1.3.4. Degradation of miRNAs

Mainly because of their small size and by the protection RISC proteins brings, miRNAs are not easily accessible from nucleases and have the reputation of being stable. In spite of the difficulty in evaluating the half-life of miRNAs, some studies investigated them and their effective stability. MP. Gantier and colleagues followed the lifespan of several miRNAs following *Dicer1* ablation in mice. Briefly, the authors estimated the average half-life of miRNAs of 115h (about five days) (varying from 100h for let-7b and miR-155 to 225h for miR-125b), 10-fold higher than the average mRNA half-life [93].

However, actual degradation of miRNAs first lies in its destabilization. miRNAs can be rapidly destabilized during changes of cell environment and behavior such as cell cycle [94], luminosity [95] or hormonal influence [96]. As detailed in paragraph 1.3.2.1, 5' or 3' unprotected ends results in a rapid decay of mRNAs. The same process is observed in miRNAs. In the case where 5' or 3' ends of the miRNAs were not protected by the RISC, miRNAs are susceptible to be tagged by uridylation and to be degraded. This phenomenon can happen to the passenger strand, which is not chosen by the RISC to induce miRNA-mediated silencing. Adenylation was also found to be a stabilizer of miRNAs so adenylases could act to destabilize them before decay [97].

Nucleases attacking miRNAs were reported to be substantially the same as the ones targeting mRNAs, such as XRN2. The small RNA degrading nuclease 1 (SDN1) was also found to degrade miRNAs efficiently from 3' to 5' in *Arabidopsis thaliana* and *C. elegans* and remains to be studied in mammals [98, 99].

1.3.5. Clinical applications of miRNAs

1.3.5.1 miRNAs as biomarkers

Besides being present in cells and organs, miRNAs are detectable in body fluids such as urine, serum or saliva. Their presence in accessible body fluids and their easy quantification makes them good candidates as biomarkers. A biomarker is “any substance, structure or process that can be measured in the body or its products and influences or predicts the incidence of outcome or disease”, as defined by the World Health Organization [100]. In body fluids, miRNAs are mostly carried in exosomes [101].

Exosomes are extracellular vesicles ranging from 40 to 100 nm diameter budding from the plasma membrane of endosomes [102]. Moreover, miRNAs can also be carried by ribonucleoprotein complexes such as AGO2, making them highly resistant to ribonuclease attacks [103]. Like other endocytosis vesicles, exosomes are coated with adhesion molecules and major histocompatibility complex (MHC) molecules that are involved in antigen presentation [104].

However, a biomarker, or in this particular case, miRNAs, must be expressed differently enough between controls and patients to establish a threshold where controls cannot be considered as patients, and inversely. Knowing that miRNA fold change is generally no more than 3, except for some individual miRNAs in particular situations, miRNAs can hardly be considered as good biomarkers or diagnostic tools on their own. Still, they can be invaluable in the case of no or bad diagnostic in rare diseases such as cancers or autoimmune diseases [105, 106]. For example, miRNAs can be used to classify patients in HIV infection [107], and to monitor the evolution of a disease or the response to treatments [108].

1.3.5.2 miRNAs as therapeutic targets

Three major techniques were developed to act against miRNAs, before and after biogenesis: miRNAs sponges, antisense oligonucleotides (ASOs, or antimiRs) and pharmacological molecules [35].

miRNA sponges, first described and investigated by MS. Ebert, JR. Neilson and PA. Sharp, are designed to act as competitive inhibitors of miRNAs [109]. They are naturally present in the organism or result from artificial constructs [110, 111]. Briefly, sponges correspond to RNA sequences that display several miRNA-binding sites that will be transcribed. These sequences will attract miRNAs so that they will not interact with target mRNAs (Figure 6A). miRNA sponges have to be highly expressed under a strong promoter to achieve their function, such as U6 or CMV promoters. Naturally present miRNA sponges can be circular [111] or long non-coding transcripts and may be called competitive endogenous RNAs (ceRNAs) [112, 113]. Artificial constructs can be delivered *in vivo* by viral vectors and can be designed to be under the influence of a tissue-specific promoter (reviewed in [110]).

ASOs, also named antimiRs, may be the most intuitive approach to target miRNAs. Their mechanism consists in designing an RNA sequence complementary to a specific miRNA to neutralize it (Figure 6B). ASOs will interact with the miRNA into the RISC so that miRNAs cannot reach the target mRNAs [35]. However, unmodified ASOs are useful in *in vitro* conditions but remain difficult to exploit in *in vivo* experiments due to nucleases present in serum. 2'-O-methyl modifications were demonstrated to be the most potent way to stabilize of ASOs [114, 115] but such modified ASOs, also called antagomiRs [116] were shown to be very sensitive to nucleases [117]. Moreover, KA. Lennox and MA. Behlke concluded that locked nucleic acids (LNA)-2'OMe-phosphorothioate combination was the better alternative to a maintained efficiency *in vivo*. LNA are RNA structures where the 2'-oxygen is bound to the 4'-carbon of the ribose, making it more stable and increasing its temperature of melting (T_m) [118]. Phosphorothioate modifications consist of exchanging an oxygen by a sulfur at the phosphodiester bond between two nucleotides (Figure 6C) [117]. The first clinical trial involving ASOs was developed in 2010 to test the effect of the Miravirsen, an LNA- phosphorothioate modified ASO sequestering miR-122 in the context of hepatitis C virus infection. The authors confirmed the efficiency of the treatment, as after 14 weeks, four out of nine patients treated with the highest dose and one out of nine patients treated with a medium dose did not display any residual hepatitis C virus RNA levels and viral resistance was not seen in all patients [119]. Since then, several phase I or II clinical trials were open for other miRNAs in various disorders. It should be noted that the difference between ASOs and antimiRs is not clearly defined. R. Rupaimoole and FJ. Slack defined antimiRs as structurally similar to ASOs without being more precise [116].

Some pharmacological compounds selectively inhibit the expression of certain miRNAs, such as azobenzene for miR-21 [120] and benzimidazole for miR-96 [121]. Some compounds also act on the biogenesis pathway. For example, enoxacin enhances the production of miRNAs by acting on TRBP affinity [122]. Some other compounds can be found by active screening of mature and precursors miRNA libraries (Figure 6D) [121].

Along with the development of all these techniques to act on miRNAs in order to treat pathophysiological situations, it might be wise to have wider look at manipulating miRNA expression, knowing that they are tightly regulated.

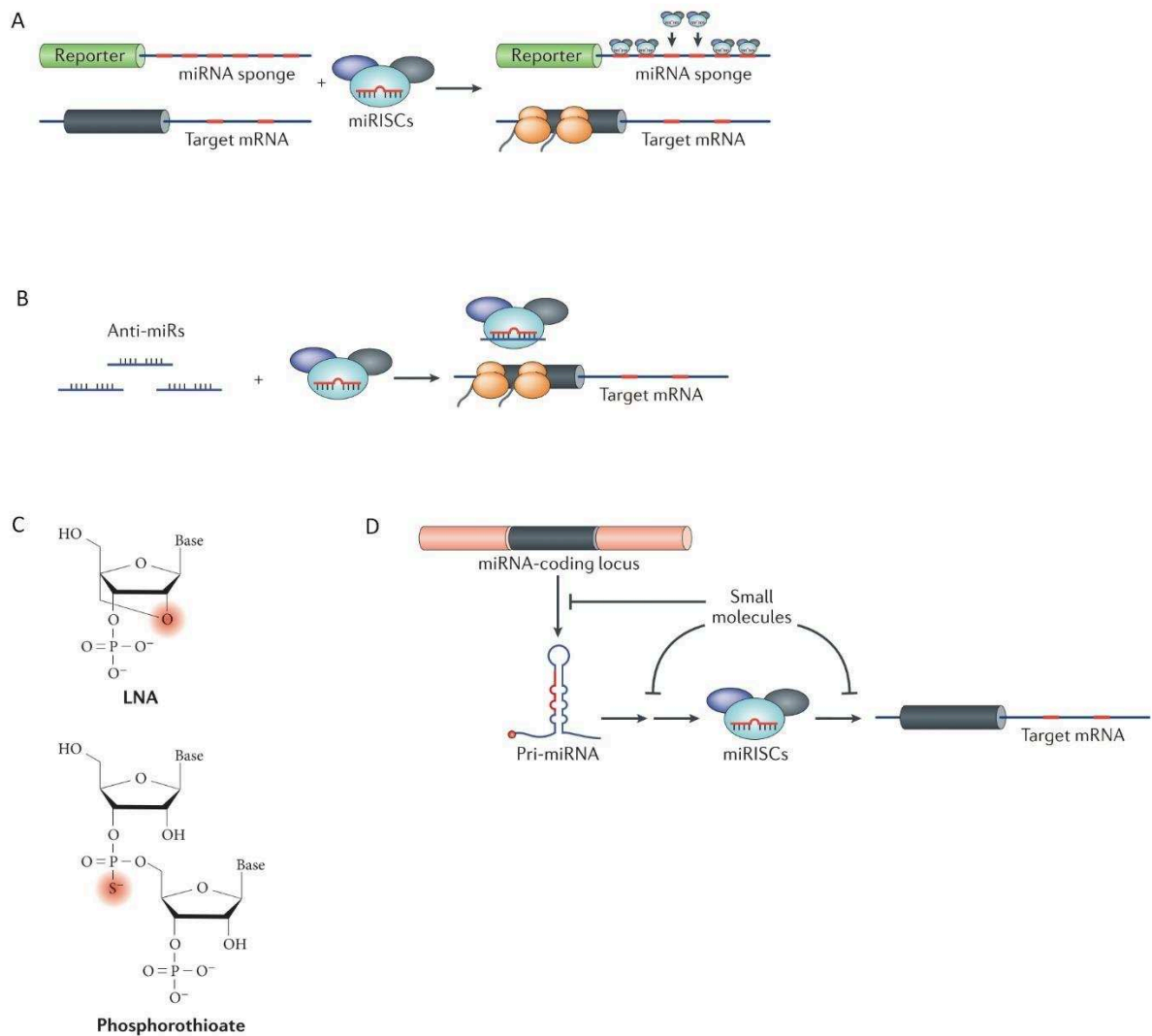


Figure 6: miRNAs as therapeutic targets

miRNA sponges (A) are expressed artificially from a reporter coding for the miRNA responsive element of a target miRNA. This way, when expressed, miRNAs will preferentially target these sponges and not their initial targets. (B) AntimiRs are unmodified ssRNAs meant to bind to the mature miRNA strand, thus leading to a functional blockade. (C) AntimiRs were chemically modified, by binding oxygen and carbon atoms of the ribose together (locked nucleic acids or LNA) or by replacing an oxygen atom by a sulfur (phosphorothioate). Finally, some pharmacological compounds are able to act on the miRNA biogenesis (D). Adapted from [35].

1.3.5.3 miRNAs as therapeutic tools

The other way to use miRNAs in a pathophysiological context is as tools to treat or to ameliorate a disorder. In pathologies, we often observe an altered expression of one or several miRNAs compared to controls. In the case of an increase, ASOs (detailed in the previous paragraph) are more suitable to adjust the miRNA level to the control one. However, in case of a decrease, we will tend to use miRNA

mimics to increase their expression level. miRNA mimics are synthetic dsRNAs designed to bypass the biogenesis pathway until the loading in the RISC and to mimic a miRNA duplex [123]. They have the advantage of being processed in part by AGO2 and to later act as endogenous miRNAs. Contrary to ASOs, if they are too much modified, they will not be taken up by the RISC and not be efficient. However, to prevent degradation *in vivo* by nucleases, miRNA mimics are surrounded by modified liposomes: stable nucleic acid-lipid particles (SNALPs). However, cargo delivery *in vivo* has yet to be improved [124]. In serum and other biofluids, miRNAs are very stable. Knowing that, teams succeeded in modulating miRNAs in exosomes *ex vivo* or to address a specific type of exosomes-carrying miRNAs to particular cells for them to deliver their cargo very specifically [125, 126]. Combining these two approaches could be very promising, for example by bringing miRNAs to an organ lacking these miRNAs. An approach using miRNAs to limit the expression of genes, in a specific cell context, was investigated by Boisgerault *et al.* [127]. The authors used in their advantage the restricted expression of miR-142-3p in immune system cells to improve the transduction efficiency of their transgene and prevent an immune response towards the transgene. Briefly, they designed a vector with miR-142-3p target sequences coded with their transgene of interest. Therefore, when injected systemically, miR-142-3p will be fixed on the transcribed designed target sequences and prevent the expression of the transgene in immune cells, thus preventing an immune response.

2. Myasthenia Gravis

The class of myasthenic syndromes gathers autoimmune Myasthenia Gravis (MG), the Lambert-Eaton myasthenic syndrome and congenital myasthenic syndromes. They are all characterized by defects at the neuromuscular junction (NMJ) but differ in terms of mechanisms.

Autoimmune Myasthenia Gravis (MG) is a rare autoimmune disorder, characterized by the presence of antibodies directed against several components of the post-synaptic membrane of the NMJ, leading to an impaired transmission signal to the skeletal muscle. This results in a generalized fatigability during effort, sometimes affecting respiratory and swallowing muscles. The Lambert-Eaton myasthenic syndrome is also an autoimmune disorder characterized by defects at the presynaptic membrane of the NMJ, caused by autoantibodies directed to voltage-gated calcium channels [128]. Congenital myasthenic syndromes are inherited disorders and must be distinguished from autoimmune MG and Lambert-Eaton Myasthenic Syndrome. Congenital myasthenic syndromes display similar symptoms as autoimmune MG, due to defects in neuronal transmission as the consequence of mutations of proteins of the NMJ.

During my PhD, I only focused on autoimmune MG; there will be no further mention of the Lambert-Eaton Myasthenic Syndrome or congenital myasthenic syndromes in this manuscript.

2.1. History of the disease: from the first cases to recent findings

There are some uncertainties concerning the first described MG case. Reportedly, Indian Chief Opechankanough, who died in 1644, seemed to have developed weaknesses making him “unable to walk” and making his “eyelids so weak they had to be held by attendants”. However, the sources came from correspondence between settlers in America and England and can be discussed [129]. In 1672, Thomas Willis published several reported cases in *De Anima Brutorum*, including the case of a woman that “speaks freely and readily enough for a while, but after a long period of speech, she is not able to speak a word and is as mute as a fish. Her voice does not return for one or two hours” [130, 131]. In the 19th century, Wilks, Erb and Goldflam described some clinical features of MG: fatigue, fluctuating

symptoms with relapses and remission cycles [132-134]. F. Jolly then showed that stimulation of the nerve innervating the patient's muscle caused reduced muscle contraction. After being named for the Erb-Goldflam disease a few times, Jolly introduced the name "myasthenia gravis pseudoparalytica" after her discovery [135]. The name can literally be decomposed as "*myo*", greek for muscle, "*asthenia*" for fatigue, "*gravis*" Latin for severe and "*pseudoparalytica*" for pseudo-paralysis. After several discoveries, such as the improvement of MG after anticholinesterase treatment [136] and the importance of acetylcholine (ACh) in the NJM, this evidence led to suggesting MG as a neuromuscular disorder. Thanks to the discovery of α -bungarotoxin, a snake toxin that binds specifically and irreversibly to AChR [137], Fambrough *et al.* showed that defects in ACh receptor (AChR) at the NMJ in MG patients may be the cause of the disease [138]. After the immunization of rabbits with purified AChR by J. Patrick and J. Lindstrom, the rabbits developed MG symptoms by producing specific antibodies to purified AChR (later called Experimental Autoimmune Myasthenia Gravis, EAMG) [139], suggesting that MG could be due to autoantibodies directed at AChR. Later on, additional evidences about the presence and the role of AChR in MG emerged: detection of anti-AChR antibodies in the serum of patients [140], transfer of human serum to mice led to the development of MG in the animals [141], improvement of symptoms after plasma exchange in patients [142] and co-localization of the lytic complement component C9 and AChR on the motor endplate [143].

For 30 years, AChR was the only antigen characterized in MG. It was only at the beginning of the 21st century that antibodies against the muscle associated receptor tyrosine kinase (MuSK) were described in 70% of seronegative MG patients [144]. Ten years later, another antigenic target was described, the low-density lipoprotein receptor-related protein 4 (LRP4), with antibodies leading to MG symptoms [145]. Several other antigens were found in MG patients such as titin [146], ryanodine [147], agrin [148, 149] and cortactin [150], but they were found in AChR⁺ and MuSK⁺ patients as well and were not exclusive to MG. The role at the NMJ of the different antigenic targets involved in MG will be further explained in paragraph 2.3.1. Major discoveries regarding MG are summed up in figure 7.

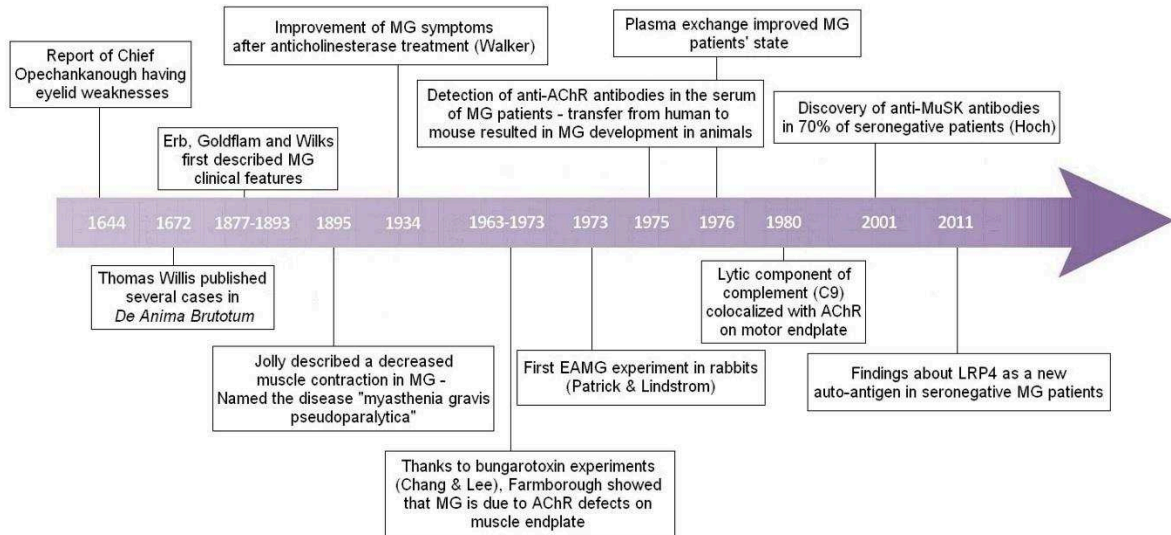


Figure 7: Major discoveries in MG

Adapted from [131].

2.2. Clinical features

2.2.1. Epidemiology

In 2010, J. McConville's group compiled 55 epidemiological studies of MG that were published between 1951 and 2004. The studies included all MG patients whatever the serotype. The incidence rate, defined by the emergence of new cases during a certain period, ranges from 1.7 to 21.3 cases per million person-years but with a high heterogeneity between studies (35 studies). However, major differences can be observed when the serotype is taken into account. In AChR⁺ and MuSK⁺ MG specific studies (eight and two studies respectively), the incidence rate ranges from 4.3 to 18.0 per million person-years in AChR⁺ MG and 0.21 per million person-years in MuSK⁺ MG.

The prevalence rate, defined by the number of cases identified at a precise point in time, ranges from 15 to 179 per million (44 studies), all MG forms included. For AChR⁺ MG, the prevalence rate ranges from 70.6 to 163.5 per million (three studies) and is 1.9 per million in the Netherlands for MuSK⁺ MG cases, the only country where the rate is known [151].

Regarding LRP4-positive MG patients (LRP4⁺ MG), 18.7% of double-seronegative patients displayed LRP4 autoantibodies (ranging from 7% to 32%) according to a study gathering 809 MG patients from

10 countries. Interestingly, the authors noticed that some AChR⁺ MG and MuSK⁺ MG patients displayed LRP4 autoantibodies, making them double-positive [152]. It is interesting that a given patient cannot be seropositive for AChR and MuSK.

All these epidemiological studies noted little global geographical variation except in Asia, where almost 50% of AChR⁺ MG cases, mostly ocular, were detected before the age of 15. Moreover, the prevalence rate may seem to have doubled between the 1960s and the 1970s and between the 1980s and the 1990s but diagnosis methods may have influenced this rate as they probably improved during these periods [153].

To sum up, AChR⁺ MG patients represent 80 to 85% of all MG patients, MuSK⁺ MG patients 5 to 4% and LRP4⁺ MG patients 2%. Remaining patients are considered seronegative [154, 155].

2.2.2. Clinical classification and associated symptoms

MG patients can be classified according to different criteria: the symptoms and the severity, the serotype, the age of onset and the implication of thymic pathologies.

2.2.2.1 Symptoms

Ocular MG

This form may affect 15 to 20% of MG patients. Ocular weaknesses symptoms can be ptosis (fall of the upper eyelid) or diplopia (double vision of a single object). Only patients without any other symptoms during the two years following their diagnosis are classified as pure ocular MG patients. Fifty percent of these patients do not display classical detectable antibodies by radioimmunoassay (RIA) but low-affinity antibodies detectable when AChR is clustered (see below) [154, 156].

Generalized MG

The Myasthenia Gravis Foundation of America (MGFA) established strict criteria to define severity grades for patients, without regarding the serotype (Figure 8). Some clinical features are common between the different MG subclasses: ptosis or diplopia (without pupillary abnormalities), weaknesses of superior limbs and neck/cervical muscles and signs of bulbar defects. Fatigue worsens after exercise

and improves after rest. However, remitting and relapsing phases can happen during the day or over long periods of time. It should be noted that MG patients do not display central nervous system dysfunctions.

Class	Clinical symptoms
I	Any ocular weakness
II	Mild Weakness. May also have ocular muscle weakness of any severity
II A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
II B	Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
III	Moderate weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
III A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
III B	Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
IV	Severe weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
IV A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
IV B	Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
V	Defined by intubation, with or without mechanical ventilation, except when employed during routine postoperative management

Figure 8: Clinical classification according to the MGFA

Symptoms are classified from class I to V, V being the more severe form of the disease. Classes II, III and IV display two subclasses (A and B) according to muscles affected. Adapted from [157].

Neonatal and juvenile MG

Pregnant MG patients can transiently induce MG to their newborns. This form of MG principally concerns AChR⁺ MG patients and results in hypotonia, respiratory troubles and impaired sucking as well as swallowing. Symptoms last from one to five months after birth, when the antibodies of the mother disappear [154].

MG in children can happen within the first years of life. Patients display AChR antibodies and mainly purely ocular symptoms. Contrary to generalized MG, there is no specific gender distribution and usually, children patients show more spontaneous remissions than adults [158]. The juvenile form of MG is predominantly found in Asians [154].

2.2.2.2 Antibodies

Antibody-specific characteristics are recapitulated in Table 1.

AChR antibodies

85% of MG patients display AChR antibodies [154]. The majority of AChR antibodies binds to α subunits of the receptor, called the main immunogenic region (MIR). They are from IgG1 and IgG3 subclasses, thus able to activate the complement cascade.

Patients can be classified in three populations, according to the age of the onset: early-onset AChR⁺ MG patients (EOMG) with symptoms before the age of 50 and late-onset AChR⁺ MG patients (LOMG) after 50 years of age. Very late onset MG patients develop symptoms after the age of 60 and the onset may be linked to the aging population. In EOMG patients, the thymus is very often affected and develops histological and functional abnormalities (more precisely described in paragraph 2.3.3). Interestingly, more than three EOMG patients out of four are female.

The LOMG form is generally associated with the development of a thymoma, a tumor of thymic epithelial cells (TECs). The thymus does not present abnormalities like in EOMG patients, and thymectomy does not improve the symptoms of MG. However, thymectomy is mandatory to remove the tumor. In this case, MG is a paraneoplastic disease.

Low-affinity antibodies / clustered AChR

Patients seronegative for AChR, MuSK and LRP4 antibodies represent between 5 and 10% of MG patients. Some of these patients may display low-affinity antibodies for these proteins, detectable only when AChR is clustered [156]. These antibodies are of the same IgG subclass as AChR antibodies, *i.e.* IgG1 or IgG3 suggesting a common etiology and pathogenesis [159, 160]. This hypothesis is confirmed by patients being similar to “classical” AChR⁺ MG patients as regards their clinical symptoms, response to treatment and thymic abnormalities [156].

MuSK antibodies

About 5% of MG patients display autoantibodies to MuSK. These patients are adults; only rare cases were described in children or elderly patients. Like with EOMG patients, most of those affected are female. MuSK⁺ MG patients do not display thymic abnormalities, thus thymectomy is not recommended. If one-third of the patients present ocular weaknesses, patients display more bulbar

weaknesses *i.e.* masticatory weaknesses, dysphonia (hoarseness), dysarthria (troubles in enunciating) and more respiratory weaknesses than EOMG patients. Overall the condition of MuSK⁺ MG patients tends to be more severe, and patients have more myasthenic crises [161].

Interestingly, antibodies against MuSK are of the IgG4 subclass. They interfere with the clustering of the receptor and induce a presynaptic effect [162]. Contrary to IgG1 and IgG3, they do not bind the complement [160].

LRP4 antibodies

LRP4 autoantibodies are found in highly variable proportions from 7 to 32% of double-seronegative MG patients. Patients display ocular or mild generalized MG and bulbar and respiratory muscles can be involved. However, due to the recent findings about LRP4 autoantibodies, the pathogenesis of LRP4⁺ MG still needs to be investigated. The involvement of the thymus in the pathogenesis of LRP4⁺ MG remains unclear but some patients display ectopic GCs like AChR⁺ MG patients [152, 155].

Other antibodies

Some other antibodies are also found in MG patients. For example, LOMG patients can display autoantibodies directed at muscle or NMJ proteins, such as ryanodine, titin, cortactin, collagen Q (CollQ) or Kv1.4 (a subunit of the voltage-gated potassium channel) [150, 163, 164].

	AChR	MuSK	LRP4	Clustered-AChR
First publication	Aharonov <i>et al.</i> , 1975	Hoch <i>et al.</i> , 2001	Higuchi <i>et al.</i> , 2011	Leite <i>et al.</i> , 2008
Frequency	~ 85%	~ 5%	~ 2%	~ 5%
Population	EOMG: ♀ > ♂ LOMG: ♀ = ♂	♀ > ♂	♀ > ♂	Unknown
IgG isotypes	IgG1, IgG3	IgG4	IgG1	IgG1
Involvement of complement	Yes	No	Yes	Yes
Correlation with severity	No	Yes	Unknown	Unknown
Pathology of the thymus	EOMG : follicular hyperplasia LOMG : thymoma	No	Unknown	Mild follicular hyperplasia

Table 1: Antibody-specific characteristics of each type of MG

Characteristics of AChR⁺, MuSK⁺, LRP4⁺ and clustered-AChR MG patients are reviewed. Adapted from [165].

2.2.2.3 Diagnosis

In case of a ptosis, the movement of the eyelid can be improved when exposed to a very cold temperature (typically an ice pack). The ice pack test is helpful to discriminate MG from other disorders involving drooping eyelids such as oculopharyngeal muscular dystrophy. Before searching for specific autoantibodies, it can be easier to inject a small dose of cholinesterase inhibitors, knowing that AChR autoantibodies are the most widespread in MG; subcutaneously or intramuscularly injected neostigmine can improve the symptoms within 15 minutes. Another method to detect specific antibodies is a blood test. Detection tests for AChR and MuSK are well developed, but an anti-LRP4 antibody test is not yet routinely available in clinical analysis laboratories. If blood tests are not sufficient to detect specific antibodies, and electromyogram can be performed to confirm the diagnosis of MG; a decrement (decreased muscular response to nerve stimulation) is observed.

A diagnosis may encounter some difficulties such as an insufficient sensitization of city doctors to MG or to a differential diagnosis orientated elsewhere such as the Guillain-Barré syndrome, amyotrophic lateral sclerosis, multiple sclerosis or oculopharyngeal myopathy [154].

2.2.3. Treatments

The usual treatments, their efficacy among different categories of patients, their modes of action and their side effects are reviewed in Table 2.

2.2.3.1 Non-immunological treatments

The first-intention pharmacological treatment for AChR⁺ MG is acetylcholinesterase (AChE) inhibitors, such as pyridostigmine. It prevents ACh degradation thus inducing the accumulation of ACh in the synapse. It allows an optimized fixation of ACh when in competition with autoantibodies directed at AChR. It is reported to be highly efficient at the beginning of the disease. Patients well aware of their disease are encouraged to adapt their doses according to relapse or remission phases [166]. Another technique consists in increasing the release of ACh presynaptically by treating patients with ephedrine or 3,4-diaminopyridine but this treatment is not as efficient as with AChE inhibitors [155].

In MuSK⁺ MG, AChE inhibitors are less effective and a close balance between benefits and side effects is necessary [161]. Side effects can come from the over-stimulation of muscarinic receptors that are not affected in MG (reviewed in paragraph 2.3.1). As muscarinic receptors are preferentially expressed in smooth muscles, intestines, the stomach and the lungs, patients can suffer from hyper sudation, increased gut motility and excessive respiratory secretions [166].

2.2.3.2 Immunosuppressive treatments

In most cases, AChE inhibitors are not sufficient to handle the disease symptoms and patients need additional treatments, such as immunosuppressive drugs. Recommended first-line treatment would be glucocorticoids (typically prednisone or prednisolone), and azathioprine in association with pyridostigmine. Azathioprine is given in association with glucocorticoids to reduce side effects linked to a prolonged glucocorticoid treatment and shows better results. Typical glucocorticoid side effects are weight gain, mood changes, stomachaches or skin rashes. Azathioprine inhibits B and T-cell proliferation, and thus improves the general state of patients. Azathioprine can take several months to act (from 6 to 12 months) making its association with glucocorticoids convenient. Indeed, when azathioprine effects are visible, glucocorticoid doses can be diminished. Mycophenolate mofetil, by acting on the same molecular mechanisms *i.e.* the inhibition of purine synthesis and on the same cell types, can be a substitute for azathioprine. Other immunosuppressive drugs such as ciclophosphamide, ciclosporine, tacrolimus and methotrexate are considered as second-line treatments.

A promising immunosuppressive drug used in other AIDs is rituximab. This chimeric monoclonal antibody targets CD20, a surface marker expressed on B cells, from pro-B to memory B cells. If plasmablasts (antibody producing B-cells) are not targeted by rituximab; it acts on their precursors, causing a loss of cells lasting up to 12 months after the first injection [167]. Rituximab was tested in two clinical trials: in refractory AChR⁺ MG patients and in a mixed cohort of AChR⁺ and MuSK⁺ MG patients [168, 169]. Both studies conclude about the efficacy of rituximab in refractory patients, as it improves clinical status of these patients.

2.2.3.3 Thymectomy

Thymectomy is the only surgical approach to treat AChR⁺ MG, especially EOMG, and thymoma-associated MG patients.

In MG patients displaying a thymoma, thymectomy prevents the spreading of the tumor but does not improve symptoms related to MG.

In EOMG patients, the thymus is the key organ in MG and displays morphological and functional abnormalities (fully described in paragraph 2.3.2.2). Thymectomy has been performed for years to treat MG patients with slowly improving symptoms. Only recently, a prospective randomized study clearly proved that thymectomy, in association with prednisone, was more beneficial to patients than prednisone alone [170]. However, thymectomy is not recommended in MuSK⁺ MG and clinicians do not have enough retrospective experience regarding LRP4⁺ MG patients.

2.2.3.4 Crisis management

Myasthenic crisis are defined as sudden worsening of weaknesses affecting respiratory and swallowing muscles. Patients need to be taken care of in intensive care units and be placed on respiratory support. Immediately following the crisis, patients are treated with intravenous immunoglobulin (IVIg) or plasmapheresis. IVIg consists of injecting a high dose of non-specific immunoglobulins which may act by blocking Fc receptors on antibodies, neutralizing autoantibodies and down-regulating B-cell responses [171]. Fractionated plasmapheresis, or plasma exchange, is a technique used to remove antibodies including autoantibodies of the patient's plasma according to different techniques. One of them consists in placing the blood through a specific affinity column. The resulting plasma is then re-injected into the patient. For the management of myasthenic crises, these techniques are equally efficient and may depend of the historical and the habits of the hospital. These treatments are given for three to six consecutive days and may be pursued for several months until remission, in combination with immunosuppressive treatments [155].

Treatments	Efficiency in :				Remarks	
	AChR	MuSK	LRP4	Clustered-AChR	Mode of action	Side effects
AChE inhibitors (Mestinon, Mytelase, Neostigmine, Pyridostigmine)	Yes	No	Yes	No	Blockade of the enzyme degrading ACh, thus prolonging ACh half-life	Gastro-intestinal disturbances (nausea, diarrhea...)
Corticosteroids (Prednisone, Prednisolone)	Yes	Yes	Yes	Yes	Calm the immune system (broad effects)	Weight gain, mood changes, stomachache, skin discomfort...
Immunosuppressors (Aziathioprine, Ciclosporin, Methotrexate, Mycophenolate mofetil, Tacrolimus)	Yes	Yes	Yes	Yes	Blockade of purine synthesis and lymphocyte proliferation	Nausea, diarrhea, leucopenia, infections, anemia
B-cell depletion (Rituximab)	Potentially	Yes	Unknown	Unknown	Neutralization of CD20 B cells (NB: Does not target antibody-producing cells)	Infections, skin rash, nausea...
Crisis management (IVIg, plasmapheresis)	Yes	Yes	Yes	Yes	Reduction of circulating pathogenic antibodies	Minor side effects (intolerance, side effects due to catheters)
Thymectomy	Yes	No	Unknown	Probably	Surgical removal of the thymoma in LOMG, removal of hyperplastic thymus in EOMG	Surgical side effects

Table 2: Current treatments for MG

Current treatments available for MG patients displaying different auto antibodies along with their mode of action and side effects.

2.3. Pathophysiology of MG

2.3.1. Consequences of the autoantibody attack at the neuromuscular junction (NMJ)

2.3.1.1 The NMJ in physiological conditions

The NMJ, or motor endplate, is the precise location where the neurotransmission between an axon of a motor neuron and the skeletal muscle takes place (Figure 9A). An electric signal, called the action potential, is generated in the brainstem of the spinal cord to the neuron cellular body and spreads along the axon. At the end of the axon, forming the presynaptic end of the NMJ, several vesicles containing the neurotransmitter ACh accumulate. ACh is synthesized in the axon from choline and acetyl-coenzyme A (acetyl-CoA) by the action of the choline acetyltransferase. The presynaptic membrane also displays voltage-gated calcium (Ca^{2+}) channels, responding to electric signals.

When the action potential reaches the end of the motor neuron, the depolarization opens the voltage-gated Ca^{2+} channels, causing the influx of Ca^{2+} ions from extracellular milieu to the inside of the neuron. This accumulation of Ca^{2+} ions induces the fusion of ACh-containing vesicles to the presynaptic membrane and ACh is released into the synaptic cleft, sized from 20 to 80 nm, to reach AChR at the

post-synaptic membrane. The post-synaptic membrane is folded to form crests and AChRs are distributed at high density ($10.000/\mu\text{m}^2$; [172]) for a better and faster transmission of the signal. The fixation of ACh on AChRs causes their activation. AChRs are voltage-gated sodium (Na^{2+}) channels, whose activation results in a Na^{2+} influx and K^+ efflux (called endplate potential) to the muscle fiber. By chain reaction, the endplate potential activates other voltage-gated Na^{2+} channels to spread the action potential all along the muscle fiber to induce muscle contraction [173, 174]. The endplate potential is generally much higher than the threshold defined to trigger an action potential, which means that there are enough ACh secreted or AChR available at the NMJ to further induce muscle contraction. This principle is called the safety factor. If the endplate potential is below the threshold, *i.e.* there is not enough ACh present (like in the Lambert-Eaton syndrome) or not enough AChR available (like in MG), there is no muscle contraction [175].

AChR is a transmembrane pentameric glycoprotein of 290 kiloDaltons (kDa) forming a canal (Figure 9B). It is composed of one β (beta), γ (gamma) and δ (delta) subunit and two α (alpha) subunits. The γ subunit, embryonic stage specific, is replaced by a ϵ (epsilon) subunit in the adult form. Two molecules of ACh can bind the two α subunits of AChR. Four types of AChR conformation were observed: opened (following ACh or other agonists' fixation on the α subunits), resting (closed with no agonists fixed), desensitized (closed with the binding of high-affinity agonists) and inactive (slower and longer desensitized state) [176, 177].

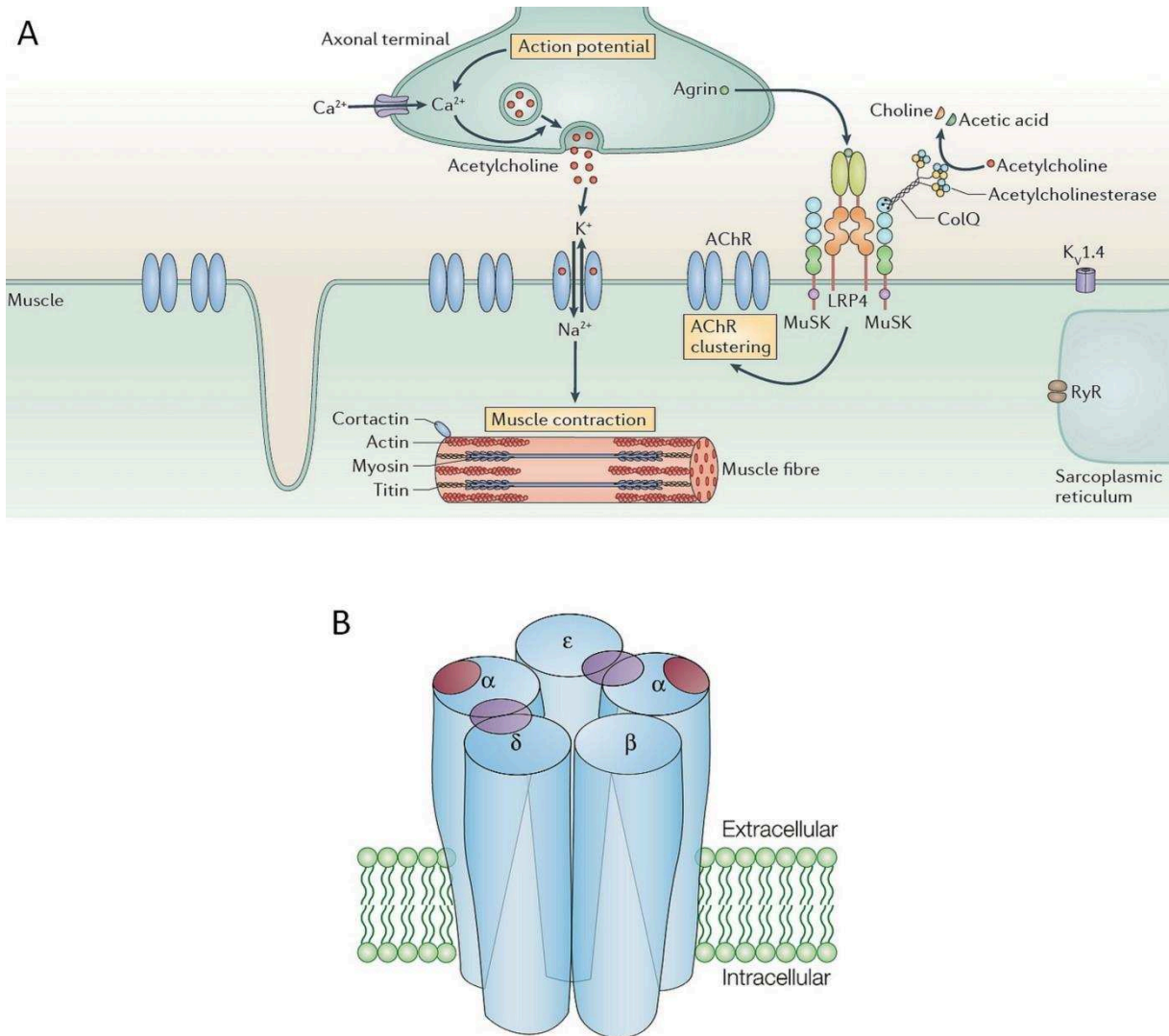


Figure 9: The neuromuscular junction

(A) The arrival of the action potential at the axon allows the release of acetylcholine at the muscle endplate. ACh binds to its receptor, on the muscle side. The fixation of ACh leads to the diffusion of the action potential throughout the muscle fiber and to muscle contraction. AChR can be clustered thanks to MuSK and LRP4 proteins. Agrin, released from the axon, binds its receptor LRP4, itself bound to MuSK. Acetylcholine esterase, also bound to MuSK via collagen Q allows the hydrolysis of ACh. (B) The AChR is a transmembrane ionotropic receptor composed of 5 subunits: $(\alpha)_2$, β , δ , ϵ . The main immunogenic regions are represented as red circles on α subunit, and bungarotoxin fixation sites are represented as purple circles. Adapted from [131, 159].

There are two types of AChRs, nicotinic and muscarinic, inducing different signaling pathways. Nicotinic receptors are ionotropic whereas muscarinic receptors are G-coupled protein receptor with seven transmembrane domains that mediate phospholipase C activation and adenylate cyclase inactivation. The receptors display different localizations and functions: nicotinic receptors can be neuronal (found in the brain and in the spine) or neuromuscular (found at the NMJ and in skeletal muscles), whereas

muscarinic receptors are found on smooth muscles in the intestine, in the lungs, in the stomach, but also in the brain [178].

Some factors are secreted from the presynaptic membrane to concentrate AChR post-synaptically in order to increase the efficiency of the signal transmission, such as agrin. Agrin is a glycoprotein acting as an inducer of AChR clustering. It binds to its receptor, LRP4, itself bound to MuSK to induce its tyrosine-mediated phosphorylation. This way, agrin indirectly induces MuSK phosphorylation [173]. Another protein necessary for the full clustering of AChR and activation of MuSK is downstream of tyrosine kinase-7 (DOK-7), an adaptor protein activating tyrosine kinase and sometimes sufficient to activate MuSK without agrin. Rapsyn is also required to induce AChR clusterization [172].

AChE is also a protein that is present in the synaptic cleft, indirectly bound to the post-synaptic membrane, to regulate secreted ACh; it hydrolyzes ACh into choline and acetic acid. AChE is carried by collagen Q, itself bound to the extracellular side of MuSK.

2.3.1.2 Pathogenic effects of MG autoantibodies

In AChR⁺ MG patients, AChR autoantibodies can act by three distinct mechanisms: degradation, internalization or blocking of the receptor (Figure 10A-C) [179].

- Degradation: AChR antibodies are from IgG1 or IgG3 subclasses, thus able to bind and activate the complement cascade. Briefly, the complement system enhances antibody responses and is involved in the removal of cells and immune complexes after an infection. The complement cascade involves more than 30 proteins found on the surface of cells or in plasma. Complement activation usually leads to cell lysis [180]. In the case of MG, post-synaptic membrane lysis and destabilization leads to AChR loss. Complement activation is the primary cause of AChR loss at the NMJ.
- Internalization: AChR autoantibodies can induce antigenic modulation. They can form a complex with AChR (cross-linking) resulting in the internalization and degradation of the cross-linked complex.

- Blocking: AChR antibodies can bind to AChR and just interfere with the binding of the ACh. This pathogenic mechanism is rarely observed in patients as they display low levels of antibodies that recognize the ACh binding site.

The antibody titer is not correlated with the severity of the disease, except in a given patient. This is due to the different antibodies and their mode of action but also to the antigenic sites. Antibodies targeting the MIR *i.e.* the α subunit are more pathogenic than the ones targeting the β subunit, suggesting that epitope patterns influence the severity of the disease [159]. Anti-AChR antibodies targeting the MIR could represent more than half of the AChR autoantibodies [181]. Interestingly, the decrease of the number of AChR at the NMJ in patients does correlate with disease severity, unlike antibody titer [159]. Progressive degeneration in MG patients is shown in Figure 10D-G.

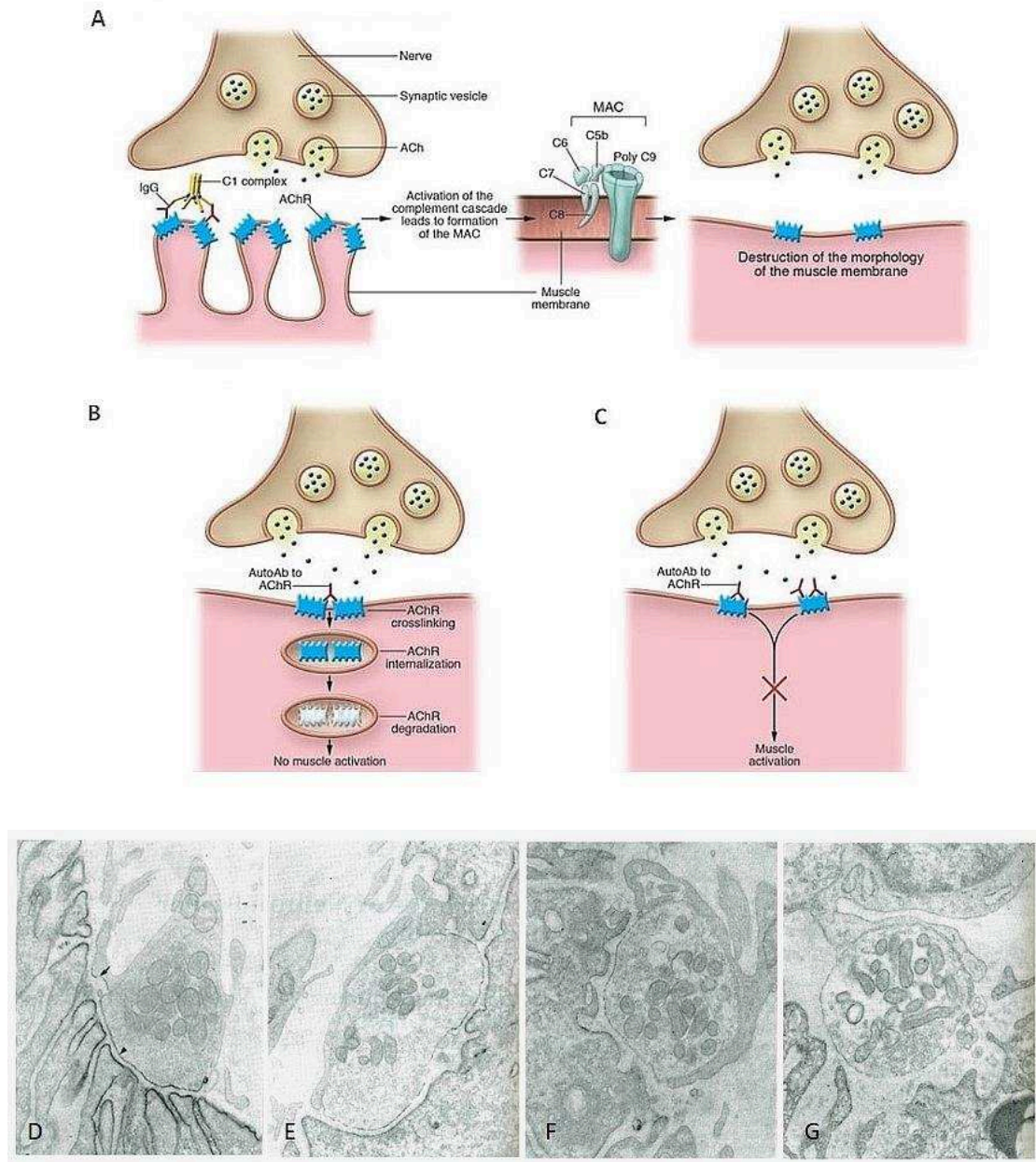


Figure 10: Mechanisms following antibody fixation on AChR

Antibodies can act by three different mechanisms: (A) degradation of the muscle membrane (activation of the complement), (B) internalization and (C) blocking (rarely observed). Compared to a control muscle (D), NMJ show progressive degeneration from mild to severe MG patients (E-G). Adapted from [179, 182].

In MuSK⁺ MG patients, unlike AChR antibodies, MuSK antibodies are from the IgG4 subclass and therefore are not able to activate complement. However, they are directly pathogenic by preventing the clustering of AChR resulting in a decreased AChR density at the NMJ.

As for LRP4⁺ MG patients, LRP4 antibodies prevent the fixation of agrin on its receptor resulting in the non-activation of MuSK. Consequences are similar to the ones observed in MuSK⁺ MG patients *i.e.* non-

clusterization of AChRs. However, LRP4 antibodies are predominantly from IgG1 and IgG2 subclasses and could induce complement activation. Pathogenic mechanisms of LRP4 antibodies still remain poorly known due to the recent discoveries of LRP4 antibodies.

In addition, MuSK and LRP4 antibodies are able to impair presynaptic differentiation as LRP4 functions as an early retrograde signal to induce presynaptic differentiation [162, 183].

2.3.1.3 Cellular and metabolic consequences of antibody attack (AChR⁺ MG)

As we do not observe muscle atrophy in MG patients, some studies investigated the cellular and metabolic consequences of antibody attack, further suggesting compensatory mechanisms.

AChR mRNA was reported to be more expressed in muscles from patients with severe MG compared to controls, as well as in muscle-cell line and control myotubes treated with anti-AChR monoclonal antibodies, suggesting a compensatory mechanism to the loss of AChR at the post-synaptic membrane [184, 185]. Moreover, using microarray experiments, Maurer *et al.* highlighted two pathways linked to AKT serine-threonine kinase (AKT): IL-6 and Insulin Growth Factor 1 (IGF-1) pathways. These observations suggest that the autoantibody attack can also activate intracellular pathways in muscle cells *in vitro* and in animal models of MG. AChR antibodies induced the overexpression of IL-6 in MG muscles or in cells treated with MG sera. In the muscle, IL-6 is known to be expressed under stress conditions and has a direct role in muscle cells destruction [186]. In addition, it was recently shown that AChR antibodies indirectly induced functional changes in satellite cells, the muscle-specific stem cells. Satellite cells from MG muscles proliferate and differentiate more than in control muscles via mechanisms involving the differentiation factor myogenin [187].

These results demonstrate that following the attack of AChR antibodies, compensatory mechanisms are involved and diverse signaling pathways are activated thus affecting muscle homeostasis.

2.3.2. Thymic abnormalities in AChR⁺ MG patients

2.3.2.1 Physiology of the normal thymus

Anatomy and cellular components of the thymus

The thymus is an asymmetrical bilobed organ located at the center of the thoracic cavity, above the heart. At birth, it weighs around 15 grams (g) and reaches its maximum at puberty, around 35g. Subsequently, the thymus starts to involute, *i.e.* a mass and size decrease, and is replaced by adipose tissue along with aging [188].

The thymus is composed of an external cortical zone (cortex) and an internal medullary zone (medulla), appearing as “dark” and “light” zones in hematoxylin-eosin staining [189]. More precisely, the thymus is mainly composed of epithelial cells (TECs) surrounded by a mesenchymal capsule to ensure its protection. Cortical and medullary TECs (cTECs and mTECs, respectively) most likely derive from a single epithelial progenitor [190-192]. Phenotypically, mTECs and cTECs are characterized by the expression of common surface markers such as MHC-II and epithelial cell adhesion molecule (EpcAM) and can be differentiated by the expression of Keratin 8 and 18 for cTECs and Keratin 5 and 14 for mTECs. TECs are the major component of the thymic stroma, *i.e.* a support tissue composed of, besides TECs, myoid cells, fibroblasts, DCs and endothelial cells [193]. Hassall’s corpuscles, structures found in human but rarely in rodents, can be found in the medulla. They are concentrically arranged cells and may be the result of highly differentiated TECs. Even if their precise role is still unclear, they are known to secrete IL-17, stromal-derived factor 1 (SDF-1, also known as C-X-C motif chemokine ligand (CXCL) 12) and TGF- α and might have a role in the generation of regulatory T (T_{reg}) cells via dendritic cells (DCs) [194].

Myoid cells display several characteristics of skeletal muscle cells, for example the expression of muscle-specific proteins such as myosin, myogenin and troponin but also AChR. Although they represent a very small cell population in the thymus, they are found in many species, suggesting an important role worth conserving among species. They act as protectors against apoptosis and

modulators of thymocyte differentiation. They might also be actors of the sensitization to AChR in MG, as they express all AChR subunits resulting in a functional AChR [195, 196].

Thymic DCs are antigen-presenting cells preferentially localized at the medulla or at the cortico-medullary junction. Contrary to “classical” DCs, which migrate to lymph nodes when they detect the presence of a microbial pathogen, thymic DCs are resident cells that live and die in the thymus. As antigen presenting cells, thymic DCs are involved in the generation of self-tolerance by inducing apoptosis in auto-reactive T-cells and the induction of T_{reg} cells (as detailed below) [194]. DCs can be divided into two subgroups: conventional DCs (cDCs) and plasmacytoid DCs (pDCs), which were named according to their phenotype, closer to the lymphoid lineage than its initial myeloid lineage. cDCs are the main population of DCs in the thymus, representing 0.3% of thymic cells. pDCs have the particularity of secreting IFN-I upon viral infection and many other cytokines [197, 198].

In addition to DCs, some other hematopoietic cells are found in low proportion in the normal thymus, such as macrophages and B cells.

Macrophages are known to act as scavengers of the environment thanks to their phagocytic activity. In the thymus, they are found in both cortex and medulla and act to phagocyte apoptotic thymocytes. Indeed, during positive and negative selection, a high number of thymocytes are discarded and undergo apoptosis. These cells are rapidly “evacuated” by macrophages [199].

Last but not least, the second major thymic population, after the TECs, are thymocytes. Thymocytes are immature T cells, deriving from hematopoietic precursor from the bone marrow and expressing C-C motif chemokine receptor 4 (CXCR4), C-C motif chemokine receptor (CCR) 7 and CCR9 allowing them to enter the thymus [200]. Thymocytes will undergo positive and negative selection to differentiate and to specialize as detailed in the following paragraph.

Functions of the normal thymus

The thymus is the organ responsible for the differentiation and maturation of T-cells. It provides a rich and complex environment so that thymocytes and stromal cells can communicate, in order to correctly undergo positive and negative selection. The thymic selection is highly stringent, as only 1 to 3% of

entering thymocytes survive to be released in the periphery [201]. The communication between thymocytes and thymic stroma mainly relies on the recognition of random peptides presented by MHC molecules on antigen presenting cells to thymocytes' T-cell antigen receptor (TCR). In 95% of the cases, the TCR is composed of α and β chains, from the superfamily of immunoglobulins, but some TCR are composed of γ and δ chains.

T-cell precursors known as T-lymphoid progenitor cells are attracted in to thymus; they enter via blood vessels and thanks to the interplay between chemokines expressed in the thymus and chemokine receptor on T-cell precursors, at the cortico-medullary junction [202]. T cells are usually characterized by the expression of the markers CD4 and CD8. The uncommitted progenitors that neither express CD4 nor CD8 are double-negative (DN). Different DN stages are characterized by the localization of cells in the thymus and the expression of the markers CD25 and CD44. Differentiating thymocytes progress through the thymus and successively acquire and lose the expression of CD25 and CD44 markers to further express CD4 and CD8 markers (Figure 11). In the cortex, thymocytes undergo a rearrangement of their DNA further coding for the β chain of the TCR. This rearrangement, called V(D)J recombination, allows a high variability in antigen recognition. Only thymocytes that successfully rearrange the region of the β chain of the TCR are allowed to pursue to the next differentiation stage. Remarkably, from the entry to the thymus to the early double positive (DP) stage, thymocytes undergo an impressive proliferative clonal expansion, where one progenitor results in one million cells [201, 203, 204].

Once halfway through their journey, DP thymocytes, will interact with cTECs via their TCR, to commit into CD4 or CD8 lineage. According to the specific recognition of the proteasome subunit beta 11 (PSMB11), complexed with MHC-I or cathepsin L and serine protease 16 (PRSS16) associated MHC-II, DP thymocytes will become simple-positive (SP) CD8⁺ or SP CD4⁺, respectively. During positive selection, thymocytes will interact with MHC-peptide complexes on cTECs and must present a sufficient affinity to antigens to continue toward the medulla. If thymocytes do not display any affinity for MHC-peptide complexes, they undergo death by neglect [205].

SP thymocytes then migrate to the medulla and interact with mTECs [206]. mTECs have the peculiarity of expressing a variety of tissue-specific antigens (TSAs) that are present in peripheral organs, so that mTECs can select and avoid the release of auto-reactive T cells in periphery. The expression of TSAs by mTECs is controlled by epigenetic factors under the influence of the autoimmune regulator (AIRE), PRDM1 (also known as BLIMP1 [207]) and Fez family zinc finger (FEZF) 2 transcription factors. Moreover, some TSAs can be transferred from dendritic cells to mTECs to expand the repertoire. If SP thymocytes display a too strong affinity to MHC-peptide complexes, they are discarded; this is the negative selection or clonal deletion [202, 204].

The negative selection displays an exception for the development of T_{reg} cells. Indeed, $CD4^+$ T cells with a higher affinity to MHC-peptide complexes than naïve T cells will express the transcription factor forkhead P3 (FOXP3) and commit to the T_{reg} lineage [208].

If the equilibrium governing the central tolerance is altered, it can lead to AIDs.

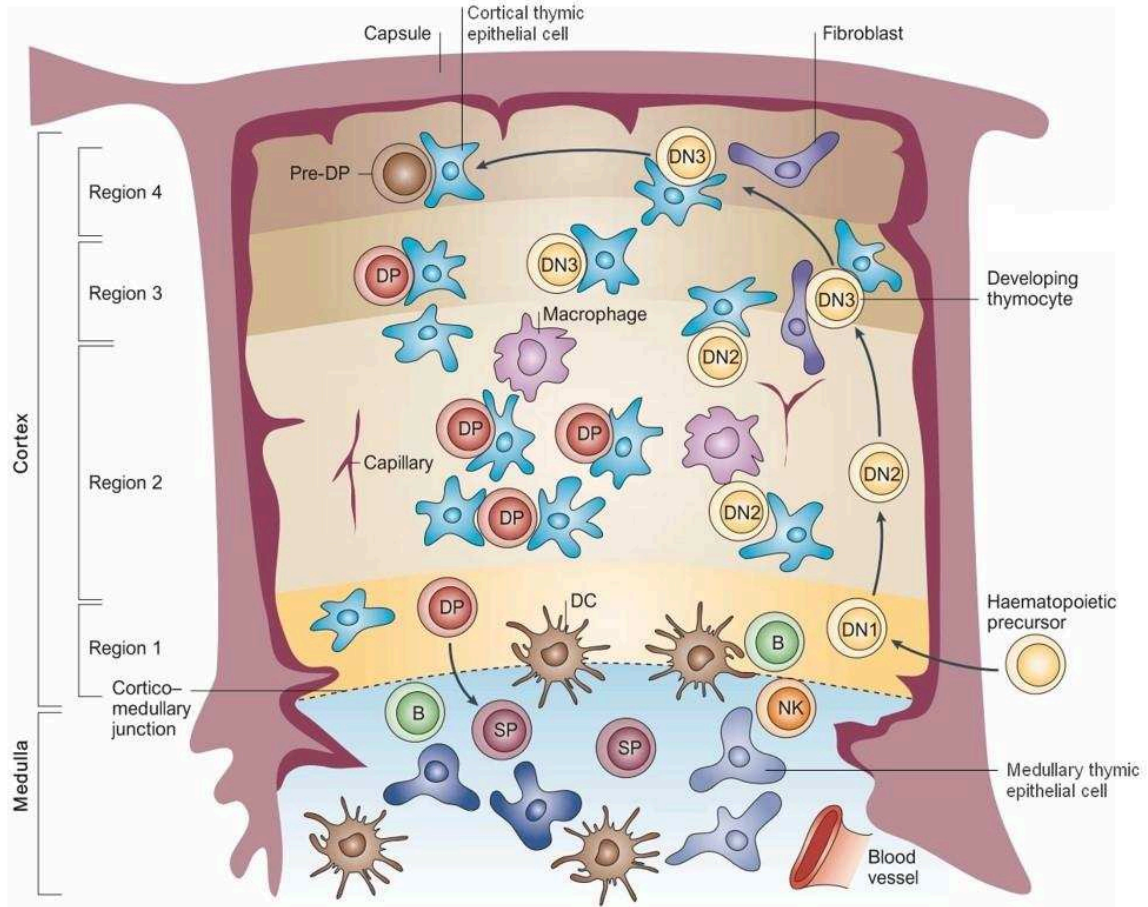


Figure 11: The thymus and the T-cell selection

The thymus is the organ orchestrating the T-cell selection. Haematopoietic precursors enter the thymus at the cortico-medullary junction and progress through the thymus to undergo differentiation steps from DN1 to DN4, starting from region 1 to 4. DN4 thymocytes, or pro-DP thymocytes, interact with cTECs and undergo the positive selection. Afterwards, thymocytes will commit to SP CD4 or CD8. SP thymocytes will progress to the medulla and T-cells bearing TCR displaying too high affinity to MHC-peptide complex will be discarded. Adapted from [209].

2.3.2.2 Cellular and molecular changes in the AChR⁺ MG thymus

Some AChR⁺ MG patients, especially LOMG patients can develop a thymoma, a tumor of TECs. Thymomas are often associated with AIDs but AChR⁺ MG remains the most associated disorder; MuSK⁺ MG associated-thymoma is very rare [210, 211]. Thirty percent of thymoma patients develop MG whereas 10 to 20% of MG patients display a thymoma. No gender predisposition but an association with HLA-A is reported [212]. It is interesting that thymomas (whether associated with MG or not) are defective in AIRE expression, potentially leading to the production of neutralizing antibodies against

IFN- α , as seen in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), characterized by mutations in the *AIRE* gene [213].

Most of patients who displays thymic abnormalities are AChR⁺ MG patients; hyperplasia from lymphoproliferative origin is observed in 50 to 60% of patients.

Active neoangiogenesis processes are observed in the thymus in EOMG patients with the development of small blood vessels, high endothelial venules (HEVs), usually found in secondary lymphoid organs (SLOs) and lymphatic endothelial vessels (LEV) [214]. The development of these vessels suggests high cell trafficking in the MG thymus thanks to the expression of specific chemokines.

Some chemokines were found overexpressed in the thymus of MG patients and could participate in the formation of ectopic GCs.

- CXCL12 (also known as SDF-1) expression is increased in the thymus of MG patients by being expressed by ectopic HEVs. B cells expressing CXCR4, CXCL12 receptor, were also found inside and around HEV [215].
- C-C motif chemokine ligand (CCL) 21 and CCL19 are two other chemokines expressed by LEV whose expression is increased in MG thymuses [214]. CCL21 and CCL19 are primarily involved in the recruitment and export, respectively, of lymphoid progenitors during thymopoiesis and in the recruitment of CCR7-expressing B cells. However, only CCL21 was found overexpressed in LEVs.
- CXCL13, one of the major B-cell attractant chemokines was found overexpressed in MG TECs [216]. CXCL13 is also able to induce the formation of GCs in an inflammatory context in EAMG mice [217].

Other chemokines not directly linked to B-cell recruitment were also found dysregulated in MG thymuses [193].

In normal thymuses, B cells represent from 1.3 to 2.6% of total thymic cells. In hyperplastic MG thymuses, they are concentrated in the medulla, representing 33% of total medulla cells [218]. They are mainly concentrated around Hassall's corpuscles. Phenotypically, they express CD19, CD20, CD22,

IgM, IgD and sometimes IgA [218, 219]. In MG thymuses, there is an infiltration of numerous B-cells that are sometimes organized in GCs (Figure 12).

GCs are transient structures where B cells highly proliferate and undergo V(D)J rearrangement and hypersomatic mutations in response to T-cell antigen presentation. These processes allow the B cells to recognize a high diversity of antigens via the B-cell antigen receptor (BCR). A high number of clones is thus generated. Cells are then selected according to their affinity for the antigen until the BCR displays perfect affinity and matching for the antigen. Next, this highly specific clone differentiates into a plasmocyte (antibody-secreting B cell) and proliferates; the resulting cells are released in periphery. In non-pathogenic conditions, GCs are induced after three to five days after T-cell mediated exposure to antigen and contact with SLOs [220]. Interestingly, ectopic GCs are found in inflamed organs in many other diseases [221].

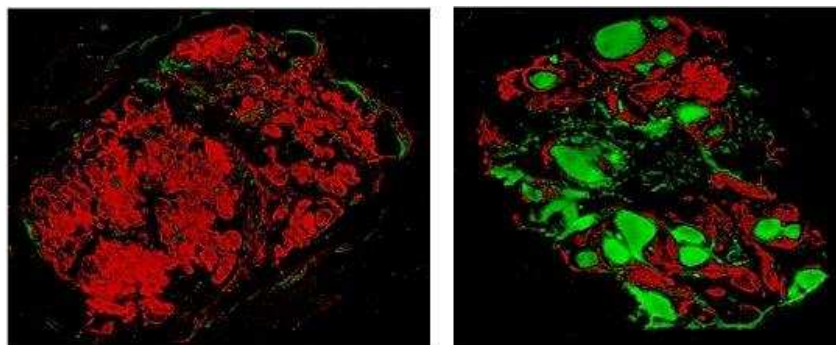


Figure 12: The thymus in MG

The thymus of AChR⁺ MG patients displays many morphological and functional changes such as abnormal B-cell infiltrations. Compared to a thymus from non-MG control (picture of a whole thymus taken with a slide scanner, left), the thymus from MG patients (right) displays B-cell infiltrates organized as germinal centers. Red: epithelial network labelled with an anti-keratin antibody; green: B cells labelled with an anti-CD21 antibody.

In MG, patients may display a variable number of GCs according to the degree of severity of the thymic hyperplasia. Moreover, it is thought that GC resolution is deficient in the thymus of MG patients [222]. Thymic hyperplasia is dependent on factors such as gender and age. Indeed, patients displaying numerous GCs (over three per section as analyzed by anatomo-pathologists), are most likely women under the age of 40. Interestingly, the gap in the gender ratio is less pronounced after the age of 50, where men develop thymic hyperplasia of lymphoproliferative origin almost as frequently as women [222]. Autoantibodies are also most likely produced, at least in part, by the B cells abnormally located

in the thymus, as antibody titer is correlated with the degree of hyperplasia [223]. Corticosteroid therapy is known to actively reduce GC number and surface and improve the general state of the patients, suggesting that GCs formation might be mediated by acute inflammation.

Altogether, these data sustain that the formation of ectopic GCs in the thymus of MG patients results from 1) an inflammatory event, 2) neoangiogenesis processes, 3) overexpression of B-cell attractant chemokines, 4) recruitment of numerous B cells in the thymus sometimes organized in GCs, 5) production of auto-reactive B cells and AChR-directed antibodies. In MG, the thymus is considered as a tertiary lymphoid organ (TLO) [224].

2.3.3. Immune dysregulation

Regarding the immune system, the pro-inflammatory and anti-inflammatory signals are balanced by specialized T-cell subsets. Pro-inflammatory T cells are a subset of CD4⁺ T cells called T helper (T_H) cells: T_H1 and T_H17. Anti-inflammatory T cells are T_H2, T follicular helper cells (T_{FH}) and T_{reg} cells. T_{reg} cells express FOXP3, a transcription factor essential to their differentiation and function. These cells play an important role in the maintenance of peripheral tolerance by calming the immune system after the resolution of a pathogen attack.

In MG, if the number of T_{reg} cells is unchanged in the thymus and in periphery, their function is altered. They do not exert their suppressive function when co-cultured with effector T cells (T_{eff}) cells and display a lower expression of FOXP3 compared to control T_{reg} cells. However, it remains unclear whether T_{reg} cells altered functions are a cause or a consequence of the deregulation of the immune system during MG development [225, 226].

Gradolatto *et al.* used microarray technology to compare gene expression levels in thymic T_{reg} and T_{eff} cells from non-MG controls and MG patients. In both T-cell subsets, IFN- γ and TNF- α expression levels were upregulated and functional analysis showed the implication of T_H1 and T_H17 subsets in pathogenic mechanisms associated with MG. Surprisingly, many genes from the IL-17 family were found upregulated in MG T_{reg} cells, compared to controls suggesting an involvement of the T_H17 subset in the pathogenesis of the disease [227]. Moreover, it was demonstrated that thymic T_{eff} cells are also

defective in MG as they are resistant to T_{reg} suppression. Interestingly, the number of T_{FH} cells is increased in the thymus of MG patients and in the periphery [228].

Altogether, these data suggest that the inflammatory environment could alter the balance between T_{reg} and T_{eff} cells (in particular T_H17 cells) and sustain the hypothesis that the inflammatory environment affects the different T-cell subsets, resulting in a loop where inflammation cannot be controlled by T_{reg} cells [165].

2.3.4. Etiological mechanisms of MG

2.3.4.1 Genetic and epigenetic predisposition

Genetic predisposition

The autoimmune form of MG is not characterized by specific mutations, unlike congenital myasthenic syndromes. However, mutations in some alleles of certain genes can increase the predisposition to MG. It is the case for HLA genes. The HLA locus is composed of eight main regions (A, B, C, DP, DM, DQ, DR and class III) coding for MHC-I or MHC-II molecules. Briefly, A, B and C regions code for MHC-I molecules while DP, DQ and DR regions code for MHC-II molecules. The HLA A1-B8-DR3-DQ2 haplotype, called AH8.1, was reported to be associated with EOMG but interestingly also with other AIDs, such as systemic lupus erythematosus and the Sjögren syndrome [229]. It is noteworthy that some other HLA associations were found in specific populations, for example in Chinese, Norwegian and Italian cohorts for LOMG, thymoma and juvenile MG [230].

Genome-wide association studies (GWAS) are observational studies aimed at identifying single nucleotide polymorphisms (SNPs) among a large cohort of patients. One of the first GWAS studies were conducted in Sweden and involved 116 MG patients and 100 non-MG controls. The authors demonstrated an association between a single nucleotide polymorphism on the tumor necrosis factor (TNF) α gene and EOMG patients with thymic hyperplasia [231]. A GWAS study held with 649 EOMG cases and 2596 healthy controls, all natives of Northern Europe showed, a strong association with the protein tyrosine phosphatase non-receptor type 22 (PTPN22) and the TNF- α interacting protein 3

interacting protein 1 (TNIP1) in addition to the HLA association, conferring a higher risk than PTPN22 [232]. Another GWAS study was led with 972 AChR⁺ MG North American patients and 1977 non-MG controls. Two new major associations were found, in addition to the known association with the HLA loci: with the TNF receptor superfamily member 11a (TNFRSF11A) and the cytotoxic T-lymphocyte associated protein 4 (CTLA4) loci. More precisely, TNFRSF11A and CTLA4 loci were only associated with LOMG cases, which displayed a higher frequency of the HLA loci compared to EOMG patients [233]. In addition, TNFAIP3 polymorphism is also associated with LOMG [234].

With a different approach, other non-HLA risk-associated genes were found in MG, such as cathepsin L2, galectin-1, VAV1 and B-cell activating factor (BAFF) [230, 235]. Moreover, an association of MG with a polymorphism in the promoter of the α subunit of the AChR was observed in some patients [236].

Epigenetic implications on MG

Epigenetics are heritable and reversible modifications facilitating or preventing gene expression without affecting DNA sequence. Studies showed that 30 to 40% of monozygotic twins were concordant for the disease *i.e.* both developed MG, a comparable rate to multiple sclerosis and systemic lupus erythematosus. This result shows that even with a similar DNA, heritable methylation status and *in-utero* growth, other environmental factors could contribute to the triggering of the disease [237]. Such factors could be histone modifications, DNA methylation, chromatin-binding proteins or non-coding RNAs such as miRNAs [238].

In MG, methylation changes were reported in MG-associated thymomas, where methylation is increased due to higher expression of methylation enzymes. Numerous small changes in DNA methylation were also shown between MG patients and controls and little enrichment in CpG islands [239].

2.3.4.2 Hormonal influence

It is widely known that gender is a bias in autoimmune disease onset [240]. Women are more prone to develop AIDs, in particular in puberty. This triggers the hypothesis that female predisposition can be explained by a high level of estrogen and a low level of testosterone.

To test this hypothesis, the involvement of the AIRE transcription factor was investigated, as it is a key factor in central tolerance and in the negative selection of T cells [241]. Mutations in the AIRE gene results in APECED, characterized by a variable phenotype of endocrine AIDs. In AIRE-mutated mice, animals display a higher susceptibility to AIDs and increased auto-reactive T cells in periphery [242]. AIRE controls the expression of some TSAs such as α -AChR and thyroglobulin, whose expression is decreased in females compared to males. Indeed, AIRE expression was shown to be increased upon androgen fixation to their receptors and decreased upon estrogen treatment. It is demonstrated that at puberty, AIRE expression is downregulated by the action of estrogens in women, which could hypothetically result in an increased export of auto-reactive T cells and further lead to the triggering of AIDs, in contrast to men, who display more “protective” androgens [242, 243]. AIRE involvement in etiology of AIDs is another brick of the wall of all environmental factors and genetic predisposition surrounding the development of these diseases.

However, pregnancy was shown to be both beneficial and deleterious to patients, due to the high level of hormones at the time. It was demonstrated that, upon hormonal influence, there is a switch between T_H1 and T_H2 responses, favoring T_H2 response. It can explain the worsening of T_H2 -dependent diseases such as systemic lupus erythematosus and improvement of T_H1 -dependent diseases such as multiple sclerosis, MG and rheumatoid arthritis [244].

2.3.4.3 Infections as possible triggers for MG

The literature is very rich regarding infections as triggers for AIDs [245], including MG. Viruses that were reported to induce MG are HIV [246], the West Nile virus [247], the human Foamy virus [248], the Hepatitis C virus [249], the poliovirus [250], the type I human T-lymphotropic virus [251], the cytomegalovirus [252] and the Epstein-Barr Virus (EBV) [253].

It is of interest that signs of poliovirus infection can be detected in MG macrophages into the thymus long after the onset of the disease [253]. It is also the case for EBV infection, as EBV small RNAs (EBERs) and EBV genes were detected in the thymic medulla and GCs of MG patients and not in control thymuses [253]. However, EBV marker detection and infection causing MG are controversial. Two other European studies reported that there was a lack of evidence regarding EBV implications and that EBV infection was not specific to MG patients [254, 255]. Serafini *et al.* later explained that technical bias (lower detection sensitivity) and differences in patients' characteristics (mild phenotype) could explain the differences between the studies [256]. Moreover, antibodies against a nuclear EBV protein (EBNA-1) were found to be more common in the serum of MG patients compared to non-MG controls [257]. As around 90% of the population is thought to be EBV-positive, it could be difficult to consider a link between EBV and MG development. In addition, it would be logical to find more signs of EBV infection in the thymus of MG patients due to the presence of B cells. Indeed, EBV tends to home in B cells and in epithelial cells during its latency phase [258]. However, all these data suggest that thymic inflammation could locally reactivate EBV leading to the maintenance of chronicity in MG.

In addition, using microarray experiments, inflammatory and IFN-induced genes, as well as IFN-I, were found upregulated in MG thymuses [259]. IFN-I is generally induced after the pathogen infection.

In MG, some evidences demonstrate the central role of IFN-I and abnormal pathogen-related signals activation. It was shown that Poly(I:C), mimicking a viral dsRNA, specifically induced α -AChR expression in TECs and which was able to induce MG in mice. More precisely, Poly(I:C)-injected mice showed muscle weaknesses, a higher AChR antibody titer and a decreased AChR density on diaphragms compared to untreated mice. In addition, the authors showed that *in vitro* Poly(I:C) induced the release of IFN-I from control TECs, especially IFN- β , and that AChR overexpression induced by Poly(I:C) was mediated by IFN- β pathway [260]. They also observed that IFN- β treatment in control TECs could induce the expression of CXCL13 and CCL21 mRNA and protein, suggesting its involvement in the recruitment of B cells in the thymus of MG patients. In addition, these changes were no longer

observed in IFNAR KO mice [261]. Altogether, these results suggest that IFN- β acts as an orchestrator of the α -AChR sensitization ultimately leading to the development of MG.

Moreover, it was shown that TLR agonists for TLR4 could induce ectopic GC-like structures in the EAMG model. TLR4 detects LPS that synergizes with Poly(I:C) to induce GC-like structures via the overexpression of CXCL13. TLR4 signaling pathway can further induce an IFN- β response [262]. These data also suggest that a viral infection leading to a disrupted feedback regulation loop of IFN- β could be the starting point of MG.

2.3.5. Alternative triggering events for MG

Iatrogenic effects

Several drugs such as D-penicillamine or IFN- α can induce a transient form of MG, even if such cases remain rare.

D-penicillamine is used as an anti-rheumatoid drug given to patients with rheumatoid arthritis and scleroderma. Approximately, 1 to 10% of patients treated with D-penicillamine for RA develop MG, mostly AChR⁺. However, a case was reported to have both serum anti-AChR and anti-MuSK antibodies [263]. Usually, patients develop an ocular MG, appearing from 2 to 12 months after the beginning of the treatment and disappearing after 2 to 6 months after the arrest of the treatment. Patients with D-penicillamine-induced MG also develop thymic hyperplasia and abnormal T cells [264], as found in idiopathic autoimmune MG. However, as in idiopathic MG, D-penicillamine-induced MG mechanisms are poorly known.

Interestingly, IFN- α and IFN- β were reported to induce or exacerbate MG in patients treated for chronic hepatitis C or MS [265-268]. It is worth noting that almost 10% of patients receiving IFN- α in chronic hepatitis C infection develop autoimmune disorders, with autoimmune thyroiditis being the most widespread. In contrast, IFN- α was reported to stop the development of the disease in the EAMG model in mice, suggesting a protection mechanism [269]. These results strengthen the hypothesis of the involvement of IFN-I in AIDs and in particular in MG.

Vitamin D

In MG, it was shown that vitamin D levels were decreased in patients' plasma and that an important dose could improve symptoms in severe refractory MG patients. Decreased vitamin D could increase the MG risk and progression [270, 271]. Moreover, vitamin D receptor agonists are able to act on CXCL10 (also known as IP-10) production. This is very interesting since in MG, CXCL10 and its receptor CXCR3 were found overexpressed in lymph nodes from EAMG mice and in muscles, PBMCs and thymus from MG patients [272]. Therefore, vitamin D could be involved in therapeutic strategies.

2.4. Animal models for MG

To better understand the pathology of MG and investigate new therapeutic approaches, animal models have been developed.

2.4.1. Induced animal models

2.4.1.1 The AChR⁺ MG model

The EAMG model is preferentially induced in rats and mice [273]. In rodents, AChR extracted from the electric organ of *Torpedo californica*, an electric ray, is emulsified in a potent adjuvant, the complete Freund's adjuvant (CFA) and injected in the foot pads and in the back of the animal. Animals will produce antibodies directed against *Torpedo californica* AChRs that will also affect their own receptors. This attack will result in muscle weakness (strength loss during a grip test), weight loss, high antibody titer against AChR and overall fatigability.

If the EAMG model recapitulates the major features of MG, it fails to recapitulate thymic abnormalities observed in EOMG patients.

2.4.1.2 The MuSK⁺ MG model

Animals were immunized with MuSK extracellular domain as a recombinant protein. Based on the same principle as the EAMG model, animals will produce anti-MuSK antibodies, initially directed against the injected recombinant protein, which will affect endogenous MuSK proteins. MuSK antibodies provoked muscle weaknesses (in skeletal muscles and bulbar muscles), weight loss and

presynaptic changes, comparable to what is observed in patients (alteration of retrograde signals on the presynaptic side and altered expression of presynaptic proteins). The model also recapitulates the decrease of AChR clustering at the NMJ [274, 275].

2.4.1.3 The LRP4⁺ MG model

Due to recent findings in LRP4⁺ MG, few models were developed. Shen *et al.* showed that LRP4 antibodies are pathogenic after injection in mice [276]. Mori *et al.* also showed that mice immunized with the extracellular domain of LRP4 had similar symptoms to MuSK-immunized mice [277].

2.4.2. The MG-NSG model

This model consists of grafting small pieces of thymic biopsies from MG patients in NOD scid gamma C KO (NSG) mice, *i.e.* immunodeficient mice lacking functional immune cells. Once grafted, the mice developed muscle weaknesses, produced antibodies against AChR and altered the expression of genes known to be deregulated in MG patients such as *BAFF*, *IFN- γ* and *TNF- α* [228, 261]. This model confirms that the thymus is the effector organ in the disease and is sufficient to induce MG symptoms in mice. The NSG-MG model is currently used to test new therapies such as mesenchymal stem cell therapy [278].

2.4.3. Other animal models for MG

As mentioned above, the EAMG model does not recapitulate all features of AChR⁺ MG patients, such as thymic abnormalities. To induce MG with thymic abnormalities, some models were developed and are meant to be combined with the EAMG model to be fully recapitulative of AChR⁺ MG. After Poly(I:C) injections, mice developed GC-like structures, overexpression of α -AChR, IFN- β and chemokines in the thymus. However, these thymic changes did not persist. The combination of Poly(I:C) with another TLR agonist, LPS, could induce persistent changes in the mouse thymus (until six weeks) [262].

3. Pathophysiological roles of miRNAs: what do we know about miRNAs and MG?

3.1. miRNAs and autoimmune diseases

Specific miRNAs can be involved in the regulation of the immune system by acting on hematopoietic stem cells and on immune cells development and function. The same miRNAs are often found at different stages of development for a specific lineage or at one precise checkpoint in all cell subsets. It is the case for miR-146a, involved in cytokine response during hematopoiesis, in the transition from mature to activated B cells and in early T-cell function but also for miR-155, involved in the GC response and in T_H17 potentiation, among other functions. Those two well-known miRNAs are often involved in pro-inflammatory pathways such as NF- κ B and their deregulation can lead to leukemia or chronic inflammation.

miR-181 has an important role in the lineage of B and T cells but also affects central tolerance by acting on TCR. Indeed, miR-181 acts as a “rheostat”, which adapts its expression to the needed signaling. In other words, if miR-181 expression is increased, TCR signaling will be potentiated through the repression of several phosphatases (PTPN22, DUSP6) whereas if miR-181 expression is decreased, a stronger TCR signal is required to ensure correct signaling: in early development of T cells, thymocytes highly express miR-181 resulting in a high sensitivity to TCR signals to undergo positive selection in the thymus [279-281].

Some miRNAs are grouped in the same region of a chromosome, called a cluster. One of the major clusters of miRNAs involved in the development of B and T cells is the cluster on chromosome 13 that comprises miR-17, miR-18, miR-19a, miR-19b-1, miR-20a and miR-92 (miR-17-92).

As mentioned above, different miRNAs act at different levels of the immune system. It is known that the immune system is tightly regulated and that small changes can have consequences and may lead to the triggering of AIDs.

The same miRNAs are often found dysregulated in many AIDs, due to their importance in the immune system [282]. Changes in the expression of miR-155 are observed in multiple sclerosis, in ulcerative

colitis, in systemic lupus erythematosus and in rheumatoid arthritis. miR-146a is also observed dysregulated in type 1 diabetes, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus [283, 284].

Nonetheless, specific dysregulated miRNAs in AIDs can be the sign of particular dysfunctions in inflammatory organs for example.

3.2. Dysregulated miRNAs associated with MG

3.2.1. miRNAs in the serum of MG patients

In 2014, two studies investigated the expression of miRNAs in the serum of MG patients for the first time [108, 285]. The group of A. Punga, which published its study first, investigated the expression of 168 miRNAs in the serum of eight early-onset AChR⁺ MG patients compared to eight non-MG controls (discovery set) using PCR arrays. The expression profile of 13 dysregulated miRNAs among the discovery set was further analyzed in the serum of 16 AChR⁺ MG patients and 16 non-MG controls (validation set). The patients did not receive any corticoid therapy. Three miRNAs were validated after a selection based on a few criteria (low hemolysis of the samples, miRNA amplification in all samples, maintenance of the dysregulated expression of the miRNA after normalization on miR-93-5p): miR-150-5p (miR-150), miR-21-5p and miR-27a-3p. These miRNAs were then investigated in the serum of AChR⁺ MG patients before and after thymectomy. Only miR-150 was found significantly downregulated in the serum of MG patients after thymectomy. The authors concluded that miR-150 could be a potential biomarker to monitor progression of the disease [108].

The group of I. Illa also investigated the expression of miRNAs in the serum of EOMG, LOMG and thymoma-associated MG patients. They analyzed 381 miRNAs in five patients in each group compared to five non-MG controls by PCR arrays. In EOMG patients, six miRNAs were validated in the validation cohort (n=17): miR-15b, miR-20b, miR-122, miR-140-3p, miR-185 and miR-192 [285].

The two teams did not identify any miRNAs in common after investigations in their validation cohort.

This observation can be explained by several causes:

- Nogales-Gadea *et al.* did not take sample hemolysis into account. However, some studies show that hemolysis could interfere with the expression of miRNAs. Indeed, erythrocytes express numerous miRNAs that will mask the expression of cellular miRNAs of interest [286].
- In the same study, some patients in the validation cohort were under corticoid and/or immunosuppressive therapy. The authors showed no significant differences in miRNA expression according to the treatment. However, patients with corticoid therapy alone seem to have a decreased miRNA expression, even if non-significant. In addition, it is not clear if the non-treated group included the non-MG controls. When all patients are pooled, this confusion may induce bias.
- I. Illa's group stated that miRNA expression did not differ before and after thymectomy. Nevertheless, it seems that, contrary to A. Punga's group, the authors did not have the serum of the same patient before and after thymectomy. It is more complicated to conclude about the impact and involvement of the thymus in the expression of a miRNA when comparing different patients.

Since 2014, A. Punga's group demonstrated that miR-150 and miR-21-5p were normalized after corticoid treatment and that thymectomy, besides improving the patients symptoms, lowered miR-150 expression in the serum of patients [287, 288]. Other miRNAs were shown to be downregulated in the serum of AChR⁺ and thymoma-associated MG patients: miR-20b [289, 290] and miR-15b [291]. It is worth mentioning that the majority of the studies were led on AChR⁺ MG patients as they are more abundant. However, a recent study showed that members of the let-7 family (let-7a-5p and let-7f-5p), miR-151a and miR-423 were enriched in the serum of MuSK⁺ MG patients [292].

3.2.2. miRNAs in circulating cells of MG patients

miRNA expression was also assessed in PBMCs from MG patients compared to non-MG controls. By microarrays, Jiang *et al.* identified the down-regulation of the let-7 family in the PBMCs of MG patients and later focused on let-7c and its impact on IL-10 [293]. With a similar approach, Liu's group focused their experiments on the down-regulation of miR-320a which induces the expression of inflammatory

cytokines [294]. Discovery cohorts showed intriguing results in both studies but only comprised of three non-MG controls and three patients whose clinical characteristics are not known. Afterwards, Lu *et al.* showed an increased miR-146a expression in MG PBMCs compared to non-MG controls and investigated its targets. They showed the involvement of the TLR4/NF- κ B pathway through miR-146a, suggesting a role of this miRNA in the activation of MG patients' B cells [295]. In addition, silencing miR-146a in EAMG mice improves the general state of the animals [296]. Wang *et al.* later showed an upregulation of miR-155 in PBMCs of no-corticoid treated MG patients and an improvement of EAMG mice after injection of an anti-miR-155 [297]. In 2016, Barzago *et al.* found an upregulation of miR-612 and miR-3654 in MG PBMCs. However, if none of the patients were under corticoid therapy (but under anticholinesterase inhibitors and immunosuppressive therapy), thymectomized and non-thymectomized patients were mixed which may have induced bias [298]. Similar to what was observed in the serum of MG patients, Liu *et al.* showed a decreased expression of the miR-15/16 cluster (containing miR-15a, miR-15b and miR-16) and investigated the impact of miR-15a on CXCL10 [299]. Regarding the impact on T_H17 cells and IL-17, Wang *et al.* showed that down-regulation of miR-145 could promote T_H17 response in EAMG rats [300]. In addition, Zhang *et al.* showed a correlation between miR-181c expression and IL-17 serum levels and suggested a role of this miRNA in the pathogenesis of MG, knowing the dysfunctions of T_H17 cells (see paragraph 2.3.3) [301].

Interestingly, specific immune miRNAs such as miR-155 and miR-146a are dysregulated in MG PBMCs and in other AIDs [282]. As mentioned above, miR-155 and miR-146a are involved in early differentiation stages of immune cells. It is not surprising that they are found to be dysregulated in AIDs, knowing the many deregulations of the immune system in these diseases.

3.2.3. In the thymus of MG patients

3.2.3.1 miRNAs in non-pathogenic thymuses

The first experiments aiming at understanding the role of miRNAs in the thymus used knock-out (KO) mice for important proteins of the biogenesis pathway, such as DICER. Cobb *et al.* and Muljo *et al.*

showed that Dicer-specific deletion in T cells altered their differentiation but did not affect lineage commitment [302, 303]. Dicer deletion in thymocytes also affects NK T cell development as well as iNK T-cell development and function [304, 305]. Moreover, the suppressive ability was reduced in Dicer-deleted T_{reg} cells, more pronouncedly in inflammatory conditions, as recapitulated in MG which may lead to uncontrolled autoimmunity [228, 306, 307].

In Dicer-deleted mouse TECs, the thymic architecture and function were altered as well as T-cell differentiation and peripheral tolerance. Indeed, thymuses were significantly smaller than control thymuses and CD8⁺ T cells showed decreased frequency [308, 309]. Interestingly, when Dicer is deleted in TECs and in particular in cTECs, lineage commitment is altered whereas miRNA deletion does not seem to affect lineage commitment in Dicer-deleted thymocytes [302, 309]. Thymic architecture and cellularity were also altered in Dgcr8-KO mice; Aire-expressing mTECs were particularly affected [310].

3.2.3.2 miRNAs in the MG thymus

In thymoma-associated MG

A few studies investigated the involvement of miRNAs in MG-related thymomas: miR-19b could contribute to T_H17 cells development by down-regulating thymic stromal lymphopoietin (TSLP) [311], miR-125a could affect the development and function of T_{reg} cells by acting on FOXP3 [312], and miR-20b inhibits T-cell proliferation by acting on NFAT signaling pathway [290].

Interestingly, a miRnome study was led on thymic epithelial tumors, including thymomas but it is not mentioned whether patients had a concomitant autoimmune MG. It could be interesting to know retrospectively which patients with a given miRNA profile later developed MG [313].

In EOMG patients

To my knowledge, besides our own study (Article 1, PhD), only one other team investigated miRNA expression in hyperplastic thymuses from EOMG patients [314]. Surprisingly, the authors extracted numerous members of the miR-548 family, all down-regulated. In addition, we did not see many common dysregulated miRNAs between our study and theirs. Li *et al.* next focused on miR-548k and

its putative target, CXCL13, after microarray analysis. One can wonder why only miR-548k targets CXCL13 knowing that all members of the miR-548 are very closely related (a few different nt).

Interestingly, they also showed that miRNAs could be involved in thymic hyperplasia by acting on CXCL13, as we showed with CCL21 [314].

4. Conclusion and objectives

miRNAs are potent modulators of protein expression whose mechanisms are now well-known. They are transcribed by RNA polymerase II into pri-miRNAs, matured into pre-miRNAs and end up as single-stranded mature miRNAs. They act on mRNA by different mechanisms resulting in a lowered target protein expression. In physiology, miRNAs act on development and homeostasis, and are involved in cancers and autoimmunity.

MG is a rare autoimmune disease caused by the presence of antibodies targeting protein of the NMJ such as AChR, MuSK or LRP4. In AChR⁺ MG, the majority of patients display functional and morphological thymic abnormalities, leading to thymic hyperplasia and antibody production. Even if some abnormalities, such as B-cell infiltrates, can be explained (neoangiogenic processes leading to the overexpression of chemokines), triggering processes are poorly understood. We hypothesized that dysregulated miRNAs could have a role in the thymic abnormalities observed in EOMG patients.

This PhD work was aimed at better understanding the implication of miRNAs in the triggering and development of autoimmune MG. To meet this objective, I used different approaches, such as miRnome analyses, the search for a functional biomarker and involvement of miRNAs in mice.

We performed a miRnome study in the thymus of female EOMG patients compared to non-MG controls and investigated new research avenues for MG. I put forward the involvement of miR-7-5p in thymic hyperplasia, thanks to integrative analysis, highlighted a non-described inflammatory pathway in MG, linking miR-125a and WDR1 and eventually characterized miRNA clusters on chromosome X. Regarding miR-150, previously characterized as a potential biomarker in MG, we showed its involvement in the thymus of MG patients and how it could modulate its targets in peripheral cells and thus affects their behavior. Following the work of A. Liston's group on miR-29 family and thymus, we showed that miR-29 KO mice displayed a higher sensitivity to the EAMG model thus reinforcing our hypothesis about the involvement of miRNAs in the development of MG.

II. RESULTS

ARTICLE 1

Analysis of microRNAs in the thymus of Myasthenia Gravis patients opens new research avenues

Article published in 2018

Objectives

Early-onset Myasthenia Gravis (MG) patients display numerous thymic abnormalities such as neoangiogenesis, chemokine overexpression and ectopic germinal centers leading to hyperplasia of lymphoproliferative origin. Particularly, germinal centers, physiologically characterized as dynamic structures vanishing after the resolution of an infection, become resident of MG thymuses.

Here, we investigated if miRNAs, poorly studied in the field of MG, could be involved in the occurrence, development and maintenance of germinal centers, through a miRnome study and different approaches regarding analysis. First, we individually analyzed the data with the Transcriptome Analysis Console software and highlighted the role of the most down-regulated and validated miRNA in MG thymuses: miR-7-5p. We showed that it could be involved in thymic changes through its action on CCL21, a chemokine involved in the recruitment of B cells in the thymus, on mRNA and protein levels. Next, we used the web tool MAGIA2 to perform integrative analyses to establish correlations between miRNAs and mRNAs. This tool uses microarray data and compiles them to establish correlations based on matching algorithms databases such as TargetScan and Diana-microT. We showed that miR-125a-5p, a miRNA involved in inflammation, could target WDR1, a protein linked to cytoskeleton and recently linked to autoimmune and inflammatory processes. This association was never studied in MG and opens new research avenues regarding thymic inflammation. Finally, we used a χ^2 test to search for chromosomic clusters and highlighted two down-regulated miRNA clusters on chromosome X. This region also encodes for the fragile X mental retardation gene, whose expression is down-regulated in MG thymuses. In addition, this region is highly regulated by DNA methylation and more investigations are needed to evaluate its impact on the triggering of MG.

Altogether, these data open new research avenues in the field of MG.



Original article

Analysis of microRNA expression in the thymus of Myasthenia Gravis patients opens new research avenues



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ABSTRACT

In early-onset Myasthenia Gravis (MG) with anti-acetylcholine receptor antibodies, thymic abnormalities associated with ectopic germinal centers are frequent. miRNAs by acting as post-transcriptional regulators are involved in autoimmunity. To investigate the implication of miRNAs in thymic changes associated with early-onset MG, we performed a miRnome study and data were analyzed with different approaches. miRNAs of interest were further investigated by RT-PCR and transfection experiments for functional tests.

First, analyzing specific dysregulated miRNAs, we focused our attention on miR-7-5p and miR-125a-5p, and confirmed by RT-PCR their respective down- and up-regulation in MG thymuses. miR-7 was the most down-regulated thymic miRNA in MG and we observed an inverse correlation between its expression and CCL21 mRNA expression. We next showed that miR-7 down-regulation was due to thymic epithelial cells and by transfecting these cells with miR-7, we demonstrated that it controlled CCL21 release. As CCL21 is essential for germinal center development, we suggested that miR-7 could be involved in thymic changes associated with MG. miR-125a was up-regulated in MG thymuses and is of great interest as it is known to regulate FoxP3 expression, and to modulate the different inflammatory signaling pathways.

Thanks to this thymic miRnome study, we also showed the specific dysregulation of miRNA clusters. In particular, we observed that miRNAs localized at the extremity of the X chromosome were down-regulated. This effect seemed linked to their close localization to the fragile X mental retardation 1 gene (FMR1) and the DNA methylation status.

Altogether, this miRnome analysis demonstrated that specific thymic miRNAs can be associated with MG and provides novel insights into the pathogenesis of MG.

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Abbreviations: AChR, acetylcholine receptor; DGCR8, DiGeorge syndrome Critical Region gene 8; FC, fold change; FMR1, fragile X mental retardation 1; IFN-I/β, type-I interferon/interferon β; IFNAR, interferon alpha and beta receptor subunit; MG, Myasthenia Gravis; miRNA, micro-RNA; TEC, thymic epithelial cell; WDR1, WD repeat domain 1.

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1. Introduction

Acquired Myasthenia Gravis (MG) is a rare neuromuscular disease caused by autoantibodies against components of the neuromuscular junction. Over 85% of patients develop autoantibodies against the acetylcholine receptor (AChR). MG patients suffer from invalidating fluctuating skeletal muscle weaknesses. MG is a prototype autoimmune disease and if the target organ is the muscle, the effector organ is the thymus [1,2]. AChR-MG patients with an early-onset form of the disease (mostly females under 45–50 years old) show thymic abnormalities as the thymus displays the characteristics of tertiary lymphoid organs: chronic inflammation, neoangiogenic processes with high endothelial venule and lymphatic vessel development, chemokine overexpression, such as CXCL13 and CCL21, favoring peripheral-cell recruitment and leading to ectopic germinal center development [3,4]. These changes seem mainly orchestrated by the abnormal expression of interferon (IFN)- β that is overexpressed in the MG thymus [5] and this could be related to a pathogen infection [6]. Actually, the MG thymus contains all the components for the anti-AChR response: AChR expression by thymic epithelial cells (TECs) and myoid cells, presence of B cells producing anti-AChR antibodies and of anti-AChR autoreactive T cells [1].

MicroRNAs (miRNAs), by acting as post-transcriptional regulators, have a role in physiological and pathophysiological processes, including autoimmune diseases [7]. They are transcribed as primary miRNAs, cleaved into pre-miRNAs by DROSHA and its partner DGCR8 (DiGeorge syndrome Critical Region gene 8) in the nucleus and transported to the cytoplasm by the duo DICER/TRBP1. Mature miRNAs coupled with the RNA-induced silencing complex (RISC, composed among other proteins of DICER, TRBP1, AGO2...) are guided to mRNA targets, leading to their degradation or to the inhibition of their translation, according to the perfect or imperfect miRNA-mRNA matching, respectively [8]. As a given miRNA can have several targets and a mRNA can be targeted by several miRNAs at the same time, regulation by miRNAs is very subtle and complex to apprehend [9]. The level of cytoplasmic miRNAs available to regulate mRNA expression is dependent on different regulation processes, as detailed by Gulyaeva and Kushlinskiy [10].

A few studies have investigated the global role of miRNAs in normal thymuses. In conditional knockout (KO) mice for *Dicer* or *Dgcr8* in TECs, a profound disruption of the thymic architecture and an alteration in TEC cellularity are observed. An important decrease in the number of thymocytes is also observed [11,12]. The lack of mature miRNAs in TECs can affect thymopoiesis [13], the expression of the autoimmune regulator (AIRE) and of tissue-specific antigens [14]. Surprisingly, in conditional *Dicer*-TEC KO mice, epithelial voids appear due to epithelial apoptosis and an unusual presence of CD19+ B cells is observed [11]. Specific miRNAs can also play key roles in thymus homeostasis and thymopoiesis and their regulation must be tightly controlled. Environmental factors, such as infections, are able to modify the expression of miRNAs and eventually alter the thymic function [15,16]. For example, in mice miR-29a is an important regulator of the type I IFN (IFN-I) signaling pathway by targeting a subunit of its receptor (IFNAR1) in TECs

and by reducing cell-sensitivity to IFN-I and consequently to pathogen infections [11].

We then hypothesized that dysregulated miRNAs could play a role in thymic changes leading to MG. By investigating for the first time the global expression of miRNAs and clusters of miRNAs in the thymus of early-onset MG patients, we bring new knowledge in post-transcriptional mechanisms associated with thymic changes in MG and opens new research avenues on myasthenia gravis.

2. Materials and methods

2.1. Patient selection and thymic samples

Thymic biopsies were collected from early-onset MG patients undergoing thymectomy and healthy donors undergoing cardiovascular surgery at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France). Briefly, MG and control thymuses from female donors were used for the miRnome study and RT-PCR (controls (n = 6, 15–33 years old); untreated MG (n = 12) and corticoid-treated MG (n = 8) patients were detailed in Table 1). MG and control thymuses from female and male donors were used for thymocytes extraction (controls (n = 5, from 9 days to 27 years old); MG (n = 12-Table 1)) and for TEC cultures (controls (n = 10, 10–35 years old); MG (n = 13-Table 1)). MG patients were only treated with cholinesterase inhibitors except for a group of corticosteroid-treated patients for Fig. 4B (Table 1). Patients had no other known diseases including thymoma. All the studies on thymuses were approved by a local ethics committee (CPP, authorization number ID RCB 2010-A00250-39) and informed consent forms have been collected.

2.2. RNA preparation

Total RNA from thymic biopsies was extracted using the mirVana miRNA Isolation Kit, according to the manufacturer's instructions (Life Technologies, Villebon-sur-Yvette, France). Thymic fragments were lysed directly in the Lysis/Binding Solution provided in the kit using the FastPrep FP120 instrument (Qbiogen, Illkirch, France). RNA quality was assessed (Bioanalyzer 2100, Agilent Technologies, Les Ulis, France) and samples with a RNA Integrity Number (RIN) over 7 were used in our experiments.

2.3. miRNA profiling

Global miRNA expression analyses were performed using the Affymetrix GeneChip miRNA 3.0 Array (Santa Clara, USA). Chips contained 3391 human miRNAs (1658 pre-miRNAs and 1733 mature miRNAs) and 2216 human snoRNAs, CDBox RNAs, H/ACA Box RNAs and scaRNAs. Briefly, 1 μ g of total RNA was labeled using the flashTag Biotin HSR RNA Labeling Kit (Affymetrix), followed by the hybridization overnight according to the manufacturer's instructions. Afterwards,

Table 1
(1) Degree of thymic hyperplasia: low hyperplasia (with 2 or fewer GCs per section) or high hyperplasia (with 3 or more GCs per section). (2) Interval in months between the onset of MG and thymectomy. (3) Myasthenia Gravis Foundation of America (MGFA) clinical classification. (4) Patients with corticoid treatment were only used for RT-PCR in Fig. 4B. NS: not specified.

Patient ID	Gender	Age (years)	Degree of thymic hyperplasia (1)	Interval onset - thymectomy (2)	MGFA score at thymectomy (3)	Corticoid treatment (4)	Cholinesterase inhibitors	Anti-AChR titer (nmol/L)	miRnome	PCR on thymic extracts	PCR on thymocytes	PCR on TECs
MG1	F	15	Low	13	III b	No	Yes	3.45		X		
MG2	F	23	Low	7	II a	No	Yes	2118.7	X	X		X
MG3	F	29	Low	7	I a	No	NS	83.7	X	X		
MG4	F	35	Low	24	IV a	No	Yes	11.1		X		
MG5	F	20	Low	6	II a	No	Yes	>100	X	X		
MG6	F	32	Low	6	II b	No	Yes	17.3	X	X		
MG7	F	29	Low	7	I a	No	NS	83.7	X			
MG8	F	19	Low	9	II a	No	Yes	21.3	X			
MG9	F	30	High	2	IV a	No	NS	>100	X	X		
MG10	F	30	High	14	I	No	NS	3180.2	X	X		
MG11	F	25	High	3	II a	No	Yes	3.21	X	X		
MG12	F	28	High	4	II a	No	Yes	60.38	X	X		
MG13	F	28	High	36	III a	No	Yes	9.7	X	X		
MG14	F	22	High	2	III a	No	Yes	264	X	X		
MG15	F	19	Low	18	III a	Yes	Yes	0.5		X		
MG16	F	33	Low	30	II b	Yes	Yes	4.33		X		
MG17	F	37	Low	6	III a	Yes	Yes	3.46		X		
MG18	F	23	Low	18	II b	Yes	Yes	NS		X		
MG19	F	26	Low	24	II b	Yes	Yes	4.4		X		
MG20	F	32	Low	9	III a	Yes	Yes	9.6		X		
MG21	F	38	Low	60	II b	Yes	Yes	21.4		X		
MG22	F	31	Low	30	II a	Yes	Yes	NS		X		
MG23	F	16	Low	24	III	No	Yes	52.8			X	
MG24	F	16	High	12	II b	No	Yes	NS			X	
MG25	F	41	High	12	II a	No	Yes	3.3			X	
MG26	F	24	High	24	II b	No	Yes	10.2			X	
MG27	F	23	Low	4	II b	No	Yes	20.2			X	
MG28	F	17	Low	7	II a	No	Yes	4.1			X	
MG29	F	26	Low	8	II b	No	Yes	9.6			X	
MG30	F	25	High	24	II b	No	Yes	23.4			X	
MG31	F	24	High	7	II a	No	Yes	9.4			X	
MG32	F	20	High	4	III	No	Yes	3.4			X	
MG33	F	20	High	6	II b	No	Yes	11			X	
MG34	F	16	Low	12	II b	No	Yes	10.3			X	
MG35	F	12	High	17	IV a	No	Yes	>100				X
MG36	F	18	Low	36	IV b	No	Yes	206.6				X
MG37	F	23	Low	2	II a	No	Yes	>100				X
MG38	F	34	High	90	NS	No	Yes	43				X
MG39	F	42	Low	48	III b	No	Yes	0.38				X
MG40	F	26	High	48	II a	No	Yes	851.9				X
MG41	M	20	Low	28	II a	No	Yes	87.6				X
MG42	M	24	Low	84	II b	No	Yes	35.3				X
MG43	F	12	Low	28	NS	No	Yes	>100				X
MG44	F	31	NS	24	NS	No	Yes	NS				X
MG45	F	37	Low	NS	NS	No	Yes	NS				X

miRNA chips were then scanned with the Affymetrix GeneChip Scanner 3000.

2.4. miRNA data analyses

The quality control of the scanned data was first estimated by confirming the order of the signal intensities of the Poly-A and hybridization controls using the Affymetrix GeneChip Expression Console. Then raw expression values were generated as “.CEL files” and “.CHP files” and were analyzed into the Transcriptome Analysis Console Software (TAC). The average for the intensity of fluorescence for each small RNA is calculated with the Tukey's Biweight method that calculates a robust average unaffected by outliers. The Tukey's Biweight average values are given in Tables 2A–B in a log₂ scale. TAC computes and summarizes a traditional unpaired One-Way Analysis of Variance (ANOVA) for each pair of condition groups. The fold changes (FCs) are given in linear values for MG versus control data. We selected small human RNAs that were dysregulated with a FC over 1.5 or –1.5 and p-value ≤ 0.05. Other miRNAs of interest could have also been extracted

if we had lowered the fold change threshold and these miRNAs can be found in the Supplemental Table S1.

To search for miRNA clusters susceptible to be dysregulated in MG patients versus controls, we compared for each chromosome the number of dysregulated miRNAs with a χ^2 test in Table 2A to the number of mature human miRNAs spotted on the Affymetrix GeneChip miRNA 3.0 Array.

2.5. Combined miRNA and mRNA data analyses

To sort out dysregulated miRNAs that could modulate gene expression in the thymus of MG patients, we have used the web resource MAGIA2 (gencomp.bio.unipd.it/magia2/) [17]. This web tool allows the analyses of miRNA and mRNA expression data for the detection of inversely correlated miRNA and mRNA pairs. The integration of target predictions with miRNA and gene expression profiles improves the detection of functional miRNA-mRNA relationships. We have used transcriptomic data obtained on thymus samples from MG patients and controls. This study has been published in 2006 [18] and data are

Table 2List of dysregulated miRNAs in MG patients. Mature miRNAs (A) and pre-miRNAs (B) with a fold change (FC) ≥ 1.5 and a p-value ≤ 0.05 were selected.

Name	Accession	MG bi-weight avg. signal (log2)	Control bi-weight avg. signal (log2)	MG/control FC (linear)	p-Value ANOVA	Chromosome	Cytogenetic band
A							
hsa-miR-4455-5p	MIMAT0018977	5.44	3.37	4.20	0.0137	4	4q35.1
hsa-miR-138-1-star	MIMAT0004607	5.28	3.59	3.22	0.0082	3	3p21.32
hsa-miR-486-5p	MIMAT0002177	11.56	9.89	3.18	0.0087	8	8p11.21
hsa-miR-4793-3p	MIMAT0019966	5.66	4.02	3.11	0.0451	3	3p21.31
hsa-miR-548w	MIMAT0015060	4.48	2.87	3.05	0.0363	16	16p12.1
hsa-miR-4495	MIMAT0019030	3.56	2.00	2.95	0.0286	12	12q23.1
hsa-miR-297	MIMAT0004450	5.63	4.19	2.72	0.0346	4	4q25
hsa-miR-486-3p	MIMAT0004762	6.43	4.99	2.72	0.0419	8	8p11.21
hsa-miR-4529-3p	MIMAT0019068	5.24	3.83	2.65	0.0088	18	18q21.2
hsa-miR-595	MIMAT0003263	4.40	3.03	2.58	0.0394	7	7q36.3
hsa-miR-548c-5p	MIMAT0004806	3.09	1.78	2.48	0.0270	12	12q14.2
hsa-miR-4710	MIMAT0019815	6.55	5.92	1.55	0.0086	14	14q32.33
hsa-miR-574-3p	MIMAT0003239	11.15	10.05	2.15	0.0010	4	4p14
hsa-miR-3148	MIMAT0015021	4.04	3.01	2.04	0.0302	8	8p12
hsa-miR-4485	MIMAT0019019	9.15	8.14	2.01	0.0436	11	11p15.4
hsa-miR-31	MIMAT0000089	10.75	9.81	1.91	0.0297	9	9p21.3
hsa-miR-4539	MIMAT0019082	7.68	6.89	1.72	0.0118	14	14q32.33
hsa-miR-3177-3p	MIMAT0015054	5.24	4.47	1.71	0.0397	16	16p13.3
hsa-miR-502-3p	MIMAT0004775	9.76	9.01	1.67	0.0451	X	Xp11.23
hsa-miR-612	MIMAT0003280	3.82	3.11	1.64	0.0277	11	11q13.1
hsa-miR-668	MIMAT0003881	3.92	3.21	1.64	0.0425	14	14q32.31
hsa-miR-2278	MIMAT0011778	3.98	3.32	1.58	0.0451	9	9q22.32
hsa-miR-760	MIMAT0004957	4.72	4.08	1.56	0.0318	1	9q22.32
hsa-miR-4538	MIMAT0019081	6.24	5.12	2.16	0.0114	14	14q32.33
hsa-miR-744-star	MIMAT0004946	2.09	2.67	-1.50	0.0301	17	17p12
hsa-miR-181d	MIMAT0002821	9.12	9.71	-1.50	0.0198	19	19p13.13
hsa-miR-1273e	MIMAT0018079	3.65	4.24	-1.51	0.0067	17	17q23.3
hsa-miR-3065-5p	MIMAT0015066	1.82	2.41	-1.51	0.0197	17	17q25.3
hsa-miR-642b	MIMAT0018444	6.12	6.71	-1.51	0.0113	19	19q13.32
hsa-miR-551a	MIMAT0003214	2.10	2.72	-1.54	0.0168	1	1p36.32
hsa-let-7 g	MIMAT0000414	11.47	12.11	-1.56	0.0460	3	3p21.1
hsa-miR-337-5p	MIMAT0004695	5.90	6.55	-1.57	0.0452	14	14q32.2
hsa-miR-101-star	MIMAT0004513	2.98	3.64	-1.58	0.0364	1	1p31.3
hsa-miR-376a	MIMAT0000729	2.03	2.71	-1.61	0.0233	14	14q32.31
hsa-miR-4510	MIMAT0019047	4.53	5.24	-1.63	0.0110	15	15q14
hsa-miR-4721	MIMAT0019835	6.71	7.43	-1.65	0.0105	16	16p11.2
hsa-miR-378d	MIMAT0018926	7.14	7.87	-1.67	0.0328	4/8	4p16.2/8q22.1
hsa-miR-487a	MIMAT0002178	2.39	3.16	-1.71	0.0166	14	14q32.31
hsa-miR-98	MIMAT0000096	5.83	6.61	-1.72	0.0091	X	Xp11.22
hsa-miR-363	MIMAT0000707	9.81	10.60	-1.73	0.0132	X	Xq26.2
hsa-miR-218	MIMAT0000275	2.39	3.25	-1.81	0.0466	4/5	4p15.31/5q34
hsa-miR-20b-star	MIMAT0004752	5.99	6.85	-1.81	0.0098	X	Xq26.2
hsa-miR-421	MIMAT0003339	6.79	7.68	-1.85	0.0405	X	Xq13.2
hsa-miR-1	MIMAT0000416	1.98	2.88	-1.86	0.0000	20/18	20q13.33/18q11.2
hsa-miR-148a	MIMAT0000243	8.29	9.26	-1.95	0.0092	7	7p15.2
hsa-miR-4500	MIMAT0019036	2.21	3.19	-1.98	0.0114	13	13q31.2
hsa-miR-892b	MIMAT0004918	2.83	3.82	-1.99	0.0073	X	Xq27.3
hsa-miR-379	MIMAT0000733	6.86	7.93	-2.10	0.0479	14	14q32.31
hsa-miR-34b-star	MIMAT0000685	3.66	4.84	-2.26	0.0357	11	11q23.1
hsa-miR-375	MIMAT0000728	6.54	7.73	-2.27	0.0448	2	2q35
hsa-miR-509-3p	MIMAT0002881	2.10	3.28	-2.27	0.0079	X	Xq27.3
hsa-let-7f	MIMAT0000067	10.49	11.70	-2.32	0.0205	9/X	9q22.32/Xp11.22
hsa-miR-720	MIMAT0005954	7.21	8.44	-2.34	0.0186	3	3q26.1
hsa-miR-133b	MIMAT0000770	2.61	3.95	-2.52	0.0483	6	6p12.2
hsa-miR-196b	MIMAT0001080	6.87	8.23	-2.57	0.0023	7	7p15.2
hsa-miR-890	MIMAT0004912	3.03	4.44	-2.66	0.0195	X	Xq27.3
hsa-miR-454	MIMAT0003885	5.00	6.44	-2.70	0.0334	17	17q22
hsa-miR-30e-star	MIMAT0000693	5.64	7.13	-2.80	0.0145	1	1p34.2
hsa-miR-103b	MIMAT0007402	2.43	3.97	-2.91	0.0204	5/20	5q34/20p13
hsa-miR-19b-1-star	MIMAT0004491	2.20	3.81	-3.05	0.0111	13	13q31.3
hsa-miR-7	MIMAT0000252	2.96	4.62	-3.16	0.0007	9/15/19	9q21.32/15q26.1/19p13.3
hsa-miR-429	MIMAT0001536	3.59	5.34	-3.37	0.0305	1	1p36.33
B							
hp-hsa-mir-1915	MI0008336	4.86	4.00	1.81	0.0304	10	10p12.31
hp-hsa-mir-339	MI0000815	5.06	4.23	1.78	0.0346	7	7p22.3
hp-hsa-mir-4539-star	MI0016910	6.14	5.46	1.60	0.0061	14	14q32.33
hp-hsa-mir-4638	MI0017265	2.97	2.21	1.70	0.0486	5	5q35.3
hp-hsa-mir-4730	MI0017367	4.69	3.92	1.70	0.0417	17	17q25.3
hp-hsa-mir-4776-2	MI0017420	3.96	3.22	1.67	0.0035	2	2q34
hp-hsa-mir-4539	MI0016910	5.18	4.57	1.53	0.0153	14	14q32.33
hp-hsa-mir-4516	MI0016882	3.23	2.59	1.56	0.0160	16	16p13.3

(continued on next page)

Table 2 (continued)

Name	Accession	MG bi-weight avg. signal (log2)	Control bi-weight avg. signal (log2)	MG/control FC (linear)	p-Value ANOVA	Chromosome	Cytogenetic band
hp-hsa-mir-548w	MI0014222	2.42	1.79	1.54	0.0028	16	16p12.1
hp-hsa-mir-4538	MI0016909	5.45	4.67	1.72	0.0283	14	14q32.33
hp-hsa-mir-30e-star	MI0000749	2.79	3.39	-1.52	0.0173	1	1p34.2
hp-hsa-mir-363	MI0000764	4.20	4.83	-1.55	0.0047	X	Xq26.2
hp-hsa-mir-320e	MI0014234	3.41	4.16	-1.69	0.0223	19	19q13.32

"MG" stands for Myasthenia Gravis, "Control" stands for adult controls, "MG/control" stands for the fold change for the expression level for each miRNA in MG patients versus controls.

available on the ArrayExpress database – accession no. E-MEXP-518 (www.ebi.ac.uk/arrayexpress).

For the mRNA matrix, we uploaded dysregulated gene transcripts with a FC of 1.8 and a p-value under 0.05 (97 up- and 154 down-regulated mRNAs) [18]. For the miRNA matrix, as fold changes were less discriminative, all mature miRNA (1733) expression data were uploaded. For non-matched miRNA and mRNA expression data, a meta-analysis based on the inverse χ^2 distribution identified oppositely variable miRNA-target gene pairs. Two target prediction databases that are regularly updated, were used with a medium stringency: 1) Targets can predicts miRNA targets by searching for conserved and non-conserved 8- to 6-mer sites in the 3'UTR of protein-coding transcripts [19], 2) DIANA-microT identifies miRNA targets in the 3'UTR but also in the coding sequence of protein-coding transcripts [20].

2.6. Retro-transcription and polymerase chain reaction (RT-PCR)

RT-PCR on mRNA were performed as previously described [5]. miRNAs were retro-transcribed using TaqMan® MicroRNA RT Kit (Life Technologies) according to the manufacturer's instructions. PCR reactions were carried out on a Light Cycler 480 (Roche, Meylan, France) using the TaqMan® Universal Master Mix II, no UNG (Life Technologies). The PCR cycle conditions were as followed: a cycle of denaturation of 10 min at 95 °C, 50 cycles of amplification of 15 s at 95 °C and 1 min at 60°. miRNA expression was normalized to 28S rRNA or GAPDH mRNA expression. Primer sequences and references are listed in Tables S2A–B.

2.7. Primary TEC cultures for transfection or methylation experiments

Primary thymic epithelial cells (TECs) were cultured from thymic explants and correspond mainly to medullary TECs as previously described [21]. TECs were transfected with Lipofectamine RNAiMax (Life Technologies). Briefly, 40 pmol of miR-7-5p or scramble RNA (Eurogentec, Seraing, Belgium) were diluted in 50 μ l of X-vivo15 medium (Lonza, Switzerland). In parallel, 4 μ l of Lipofectamine RNAiMax were diluted in 50 μ l of X-vivo15 medium. Then, both solutions were mixed and incubated for 20 min at room temperature. The resulting mixes were added to cultured cells (4×10^5 cells per well) at a ratio of 1:4 of X-vivo15 medium in 12-well plates for 48 h.

For methylation experiments, TECs were seeded in RPMI containing 10% of fetal calf serum (5×10^5 cells per well) and treated after 24 h with 5 μ M of 5-Azacytidine for 48 h. TECs were then lysed for total RNA extraction.

2.8. CCL21 ELISA

CCL21 antibody (AF366, R&D Systems, Lille, France) was diluted at 1 μ g/ml in coating buffer (50 mM NaHCO₃ and 50 mM Na₂CO₃, pH 9.4) and incubated overnight at 4 °C. TEC culture medium (1.5 mg/ml of proteins) were incubated for 3 h, and subsequently, 0.25 μ g/ml of biotinylated anti-CCL21 (BAF366, R&D Systems), and streptavidin-horseradish peroxidase (Life Technologies) were added. Tetramethylbenzidine (BioLegend, United Kingdom) was used for color development and plates were read at 450 nm on a SPARK 10 M microplate reader (TECAN Life Sciences, Grödig, Austria).

2.9. Statistical analyses

In dot graphs, 2-by-2 comparisons were done using the non-parametric Mann-Whitney or Wilcoxon tests as indicated in the figure legends. For correlation analyses, significance was assessed using a Pearson's correlation test.

3. Results

3.1. Analysis of differentially expressed miRNAs in the thymus of MG patients

Thymic miRNA expression profiles were compared between MG patients and age-matched female controls. Table S1 lists all miRNAs analyzed on the Affymetrix miRNA arrays. Analyzing all human miRNAs that were dysregulated in MG thymuses (FC ≥ 1.5 and $p \leq 0.05$), we found 74 miRNAs (Table 2A–B and Fig. 1A): 61 mature miRNAs (24-up and 37 down-regulated) and 13 pre-miRNAs (10 up- and 3 down-regulated) (Table 2B). 5 of the pre-miRNAs (3 up- and 2 down-regulated) were also found dysregulated as mature miRNAs: miR-548w, miR-4538, miR-4539 and miR-30e, miR-363 (Table 2A–B).

To confirm the expression profile of the most down-regulated miRNAs (miR-429-3p, miR-7-5p and miR-1-5p) or the most up-regulated miRNAs (miR-138-1-3p, miR-486-5p and miR-4455-5p) (Fig. 1B–G), we analyzed by RT-PCR their level of expression. Although the decrease or the increase of these miRNAs were consistently confirmed, the differences between MG and controls were only significant in RT-PCR for miR-486-5p and miR-7-5p (Fig. 1H–M).

3.2. Analysis of miR-7 expression in the MG thymus

From the miRnome analysis, miR-7-5p was one of the most down-regulated miRNAs in the thymus of MG patients and we confirmed by RT-PCR its down-regulation in MG thymuses compared to controls (Fig. 1I). We then demonstrated that miR-7-5p was even more down-regulated in the thymus of patients with a high degree of thymic hyperplasia (Fig. 2A). In corticoid-treated patients, miR-7-5p was still down-regulated compared to non-MG donors but a slight increase was observed when comparing corticoid-treated patients with MG patients with a high degree of hyperplasia (Fig. 2A). To further investigate the miRnome data, we also measured the level of expression of other miR-7 members: miR-7-1-3p and miR-7-2-3p. We confirmed the specific down-regulation of miR-7-5p (the leading strand) in MG thymuses, as we did not observe any change for miR-7-1-3p and miR-7-2-3p (the passenger strands) expression in the thymus of MG and control donors (Fig. 2B–C). We next analyzed if the down-regulation of miR-7-5p was observed in the two main thymic cell types: TECs obtained from thymic explants after 7 days in culture and thymocytes freshly obtained from thymic biopsies. miR-7-5p was expressed in both cell types but very weakly in thymocytes. We observed a down-expression of miR-7-5p in TECs from MG patients compared to TECs from controls (Fig. 2D). In contrast, in freshly isolated thymic cell suspension, we observed a higher expression level of miR-7-5p in cells from MG patients compared to controls (Fig. 2E). This increase was not correlated with the presence of B cells in thymic-cell suspensions from MG patients as no correlations were observed compared to CD19 mRNA

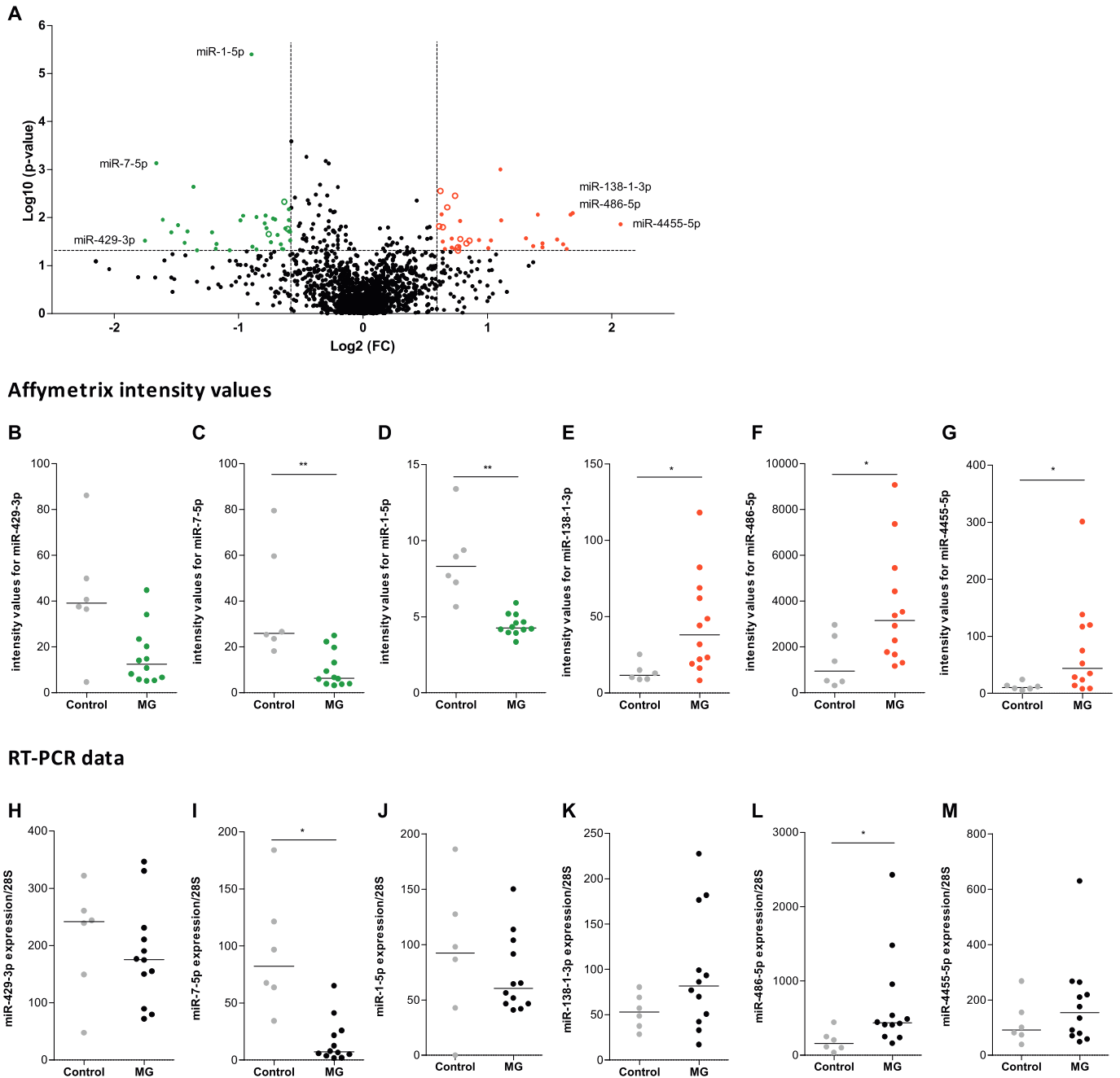


Fig. 1. Dysregulated mature and precursor miRNAs in MG thymuses compared to controls. (A) Volcano plot of differentially expressed miRNAs identified using Affymetrix GeneChip 3.0 miRNA Arrays and using the Transcriptome Analysis Console Software. Comparison of miRNAs expressed in the thymus of MG patients ($n = 12$) versus adult controls ($n = 6$). The vertical lines correspond to fold changes of 1.5 for up or down-regulated miRNAs in Log₂. The horizontal line delineates a p-value of 0.05 in Log₁₀. The red (34) and green (40) points represent the differentially expressed miRNAs with statistical significance for up- and down-regulated miRNAs, respectively. Empty dots correspond to pre-miRNAs and filled dots to mature miRNAs. Intensity of fluorescence of miR-429-3p (B), miR-7-5p (C), miR-1-5p (D), miR-138-1-3p (E), miR-486-5p (F) and miR-4455-5p (G) from the Affymetrix chips for non-MG adults (controls, $n = 6$) and MG patients ($n = 12$). RT-PCR data for miR-429-3p (H), miR-7-5p (I), miR-1-5p (J), miR-138-1-3p (K), miR-486-5p (L) and miR-4455-5p (M) in MG thymuses ($n = 12$) compared to non-MG controls ($N = 6$). miRNA expression level are given in arbitrary units and were normalized to 28S expression. p-Values were assessed by the Mann-Whitney test and only p-values < 0.05 are indicated (*p < 0.05, **p < 0.01).

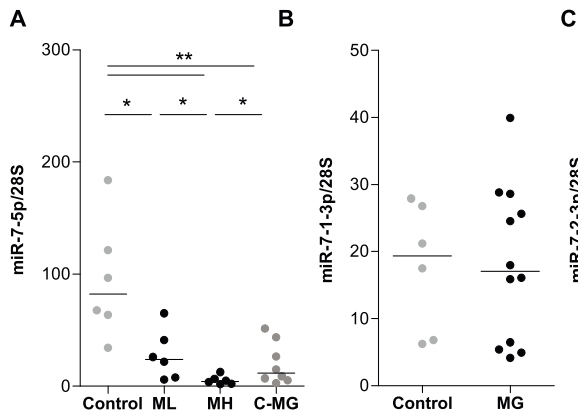
expression (data not shown). All these data confirmed that miR-7-5p was down-regulated in the thymus of MG in relation to a decrease in TECs.

3.3. Effect of miR-7 on CCL21 expression in thymic epithelial cells

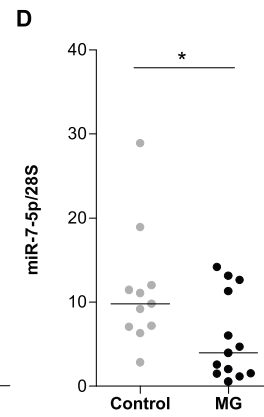
From predictive databases, we determined that miR-7-5p was of particular interest as it targets mRNAs of several genes involved in MG (Fig. 2F–G). As we observed a direct inverse correlation between miR-7-5p expression and CCL21 mRNA in the thymus (Fig. 2H), we investigated whether miR-7-5p could inhibit CCL21 expression in TECs. We

then carried out transfection experiments in TECs and analyzed the level of CCL21 released in the culture medium after 48 h. First, we demonstrated the efficiency of our transfections in primary TEC cultures by evaluating the intracellular level of miR-7-5p after transfection (Fig. 2I). Next, we demonstrated that CCL21 protein levels were decreased from 9 to 20% when TECs were transfected with miR-7-5p compared to a scramble RNA (Fig. 2J). These results sustain that miR-7 has an effect on CCL21 protein expression and a decrease in miR-7-5p expression could be responsible for the overexpression of CCL21 in MG thymuses [4].

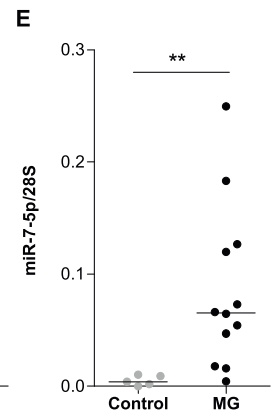
RT-PCR on thymic biopsies



RT-PCR on TECs



RT-PCR on thymocytes



F

Gene name (1)	TargetScan (2)	Miranda (3)	Diana-microT (4)
CCL21	X	X	
CXCL12	X		X
CXCL13		X	
EGFR	X	X	X
IFNAR1	X		
IFNAR2	X		
IGF1R	X		
IRF8	X		
KLF4	X	X	X
MyD88	X		
PTPN22		X	
TLR4	X	X	

G

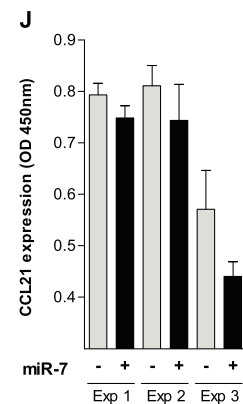
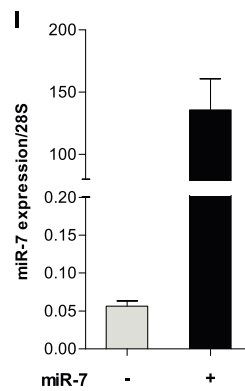
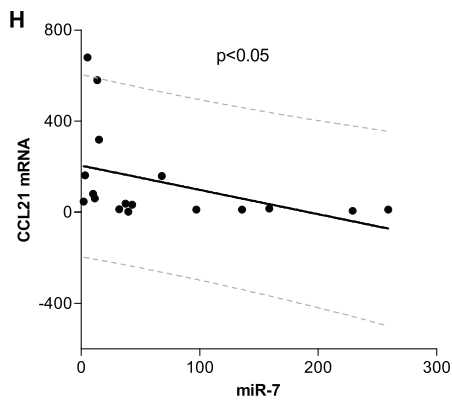
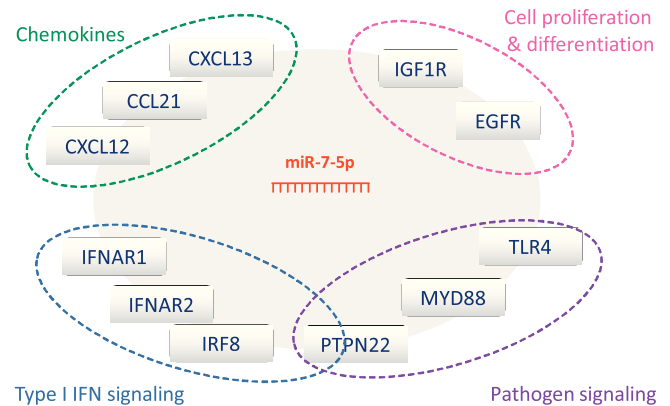
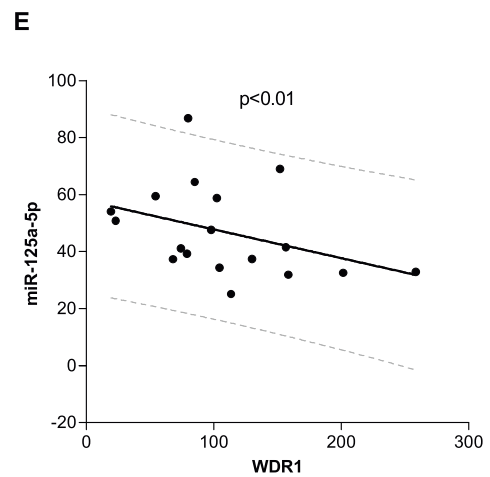
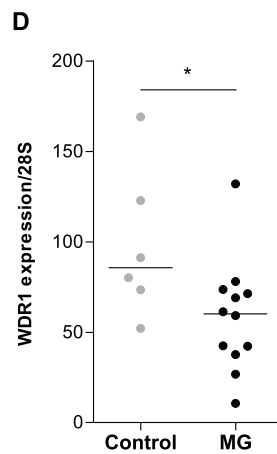
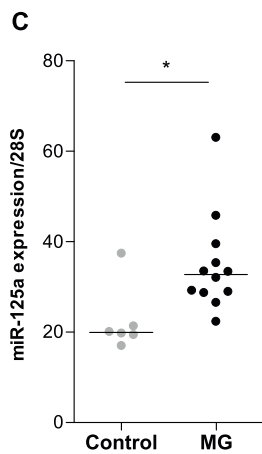
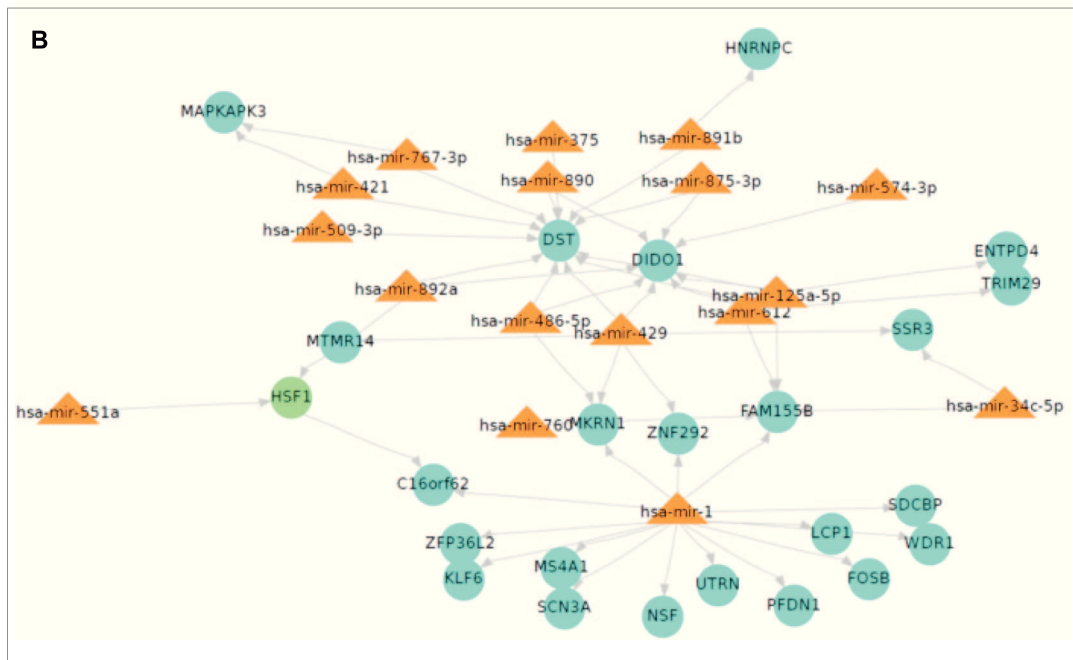
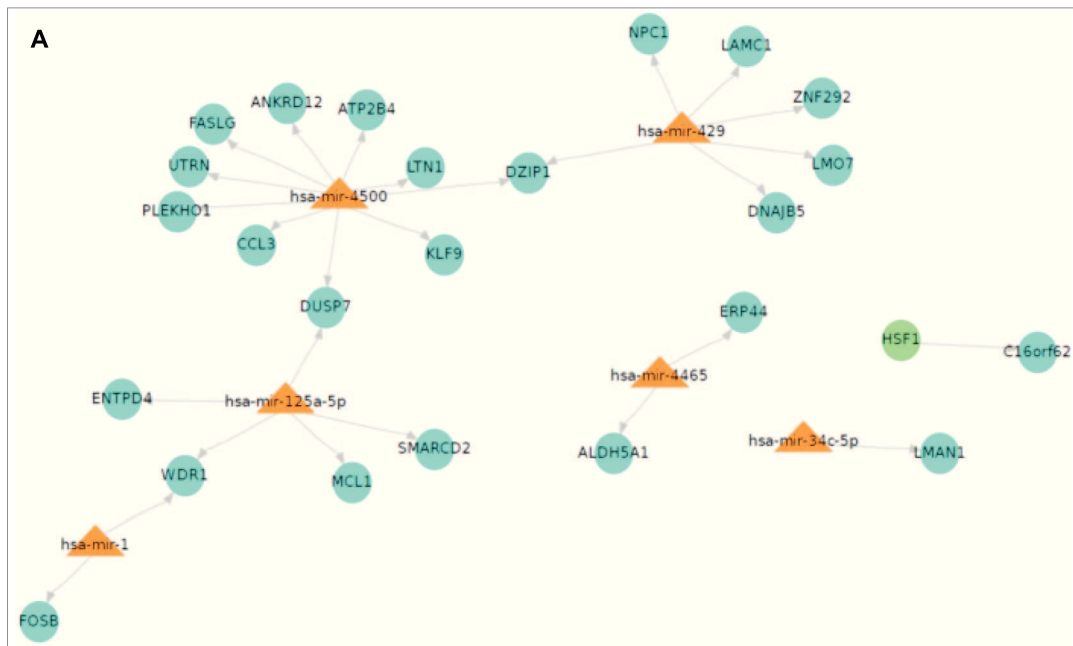


Fig. 2. Down-regulation of miR-7-5p in the thymus of MG patients. (A) PCR analyses for miR-7-5p expression in the thymus of non-MG adults ($n = 6$), of ML patients (MG patients displaying low degree thymic hyperplasia (with 2 or fewer GCs per section, $n = 6$)), MH patients (MG patients displaying high degree thymic hyperplasia (with 3 or more GCs per section, $n = 6$)) and of MG patients under cortico-therapy ($n = 8$). RT-PCR analyses for miR-7-1-3p (B) and miR-7-2-3p (C) expression in the thymus of non-MG adults ($n = 6$) and MG patients ($n = 12$). (D) PCR analyses for miR-7-5p expression in medullary TECs from non-MG adults ($n = 11$) and MG patients ($n = 13$) after 7 days in culture. (E) PCR analyses for miR-7-5p expression in freshly extracted thymic cells from non-MG controls ($n = 5$) and MG patients ($n = 14$). miRNA expression levels were given in arbitrary units and were normalized to 28S. P-values were assessed by the Mann-Whitney test and only p-values < 0.05 are indicated (* $p < 0.05$, ** $p < 0.01$). (F) List of genes dysregulated in the thymus of MG patients and targeted by miR-7-5p according to prediction databases. (1) Abbreviations: CCL21: C-C motif chemokine ligand 21; CXCL12/13: C-X-C motif chemokine ligand 12/13; EGFR: epithelial growth factor receptor; IFNAR1/2: interferon alpha and beta receptor subunit 1/2; IGF1R: insulin-like growth factor 1 receptor; IRF8: interferon regulatory factor 8; MyD88: myeloid differentiation primary response 88; PTPN22: protein tyrosine phosphatase non-receptor type 22; TLR4: toll-like receptor 4. (2) (http://www.targetscan.org/vert_71) (3) (<http://www.microrna.org>) (4) (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index). (G) Scheme for miR-7 targeted genes involved in MG and associated function. (H) Correlation between CCL21 mRNA and miR-7-5p expression in the thymus of non-MG controls ($n = 6$) and MG patients ($n = 12$). Pearson's correlation was assessed and p-value under 0.05 indicated. (I) Representative experiment of miR-7-5p level in TECs transfected with miR-7-5p (+) or with a scramble RNA (-) for 48 h. miR-7-5p expression was normalized on 28S expression. (J) CCL21 protein expression in experiments with TECs from three different donors. TECs were transfected either with miR-7-5p (+) or scramble RNA (-) for 48 h and CCL21 protein level was assessed by ELISA in the medium of TEC cultures. Error bars correspond to the measures made on three different wells.



3.4. Search for inverse correlation for miRNA and mRNA expression data

To sort out dysregulated miRNAs that could modulate gene expression in the thymus of MG patients, we have used the web resource MAGIA2 as fully detailed in the [Materials and methods section](#). We identified mature miRNAs and genes possibly regulated by transcription factors, whose expression levels were inversely correlated in the thymus of MG patients. The target prediction databases, Targetscan and DIANA-microT extracted 6 miRNAs (1 up- and 5 down-regulated) and 16 miRNAs (4 up- and 12 down-regulated), respectively. Among them, miR-486-5p was again put forward as an up-regulated miRNA in MG patients. miR-125a-5p, miR-34c-5p, miR-1-5p and miR-429-3p were identified by both predictive databases ([Fig. 3A–B](#) and [Table 3](#)).

Among these dysregulated miRNAs, we analyzed by PCR the expression of miR-125a-5p and confirmed its up-regulation in the thymus of MG patients ([Fig. 3C](#)). Among miR-125a-5p targets listed in [Table 3](#), we were particularly interested in WDR1 (WD repeat-containing protein 1), a molecule recently implicated in auto-inflammatory processes [22]. We then showed that WDR1 mRNA expression was significantly decreased in the thymus of MG patients and that its expression was indeed inversely correlated with miR-125a-5p expression ([Fig. 3D–E](#)).

3.5. Analysis of chromosomal cluster dysregulation

In the list of dysregulated mature miRNAs ([Table 2A](#)), we observed a high representation of miRNAs from the chromosomes 14 and X. Using a χ^2 test, we demonstrated for up-regulated mature miRNAs a significant overrepresentation of chromosomes 4, 8, 14 and X. For chromosomes 4 and 8, miRNAs were found up-regulated but they were spread along each chromosome ([Fig. 4A](#) and [Table 2A](#)). In contrast, for miRNAs up-regulated on chromosome 14, miR-4538-5p, miR-4539-3p, miR-4710-5p were all located in the 14q32.33 region on the negative DNA strand. Moreover, pre-miRNAs for miR-4539 and miR-4538 were also found up-regulated ([Table 2B](#)). All these miRNAs belong to a miRNA cluster well referenced in miRbase.

We found for down-regulated mature miRNAs a significant overrepresentation of miRNAs located on the negative DNA strand at the extremity of the long arm of the X chromosome ([Fig. 4A](#) and [Table 2A](#)): miR-20b-3p*, miR-363-3p and miR-509-3p, miR-890-5p and miR-892b-3p. miR-20b-3p* and miR-363-3p belong to a referenced cluster in the Xq26.2 region, while miR-890-5p, miR-892b-3p and miR-509-3p belong to two other miRNA clusters in the Xq27.3 region. These miRNAs were all found down-expressed ([Table 2A](#)). We selected 2 of these miRNAs for validation by RT-PCR: miR-20b-3p* and miR-892b-3p, and confirmed their decreased expression in MG thymuses compared to controls ([Fig. 4B–E](#)).

This chromosomal region corresponds to a fragile site where the fragile X mental retardation 1 (FMR1) gene locates. Analyzing the level of expression of FMR1 in the thymus, we observed also a significant decreased expression in MG patients ([Fig. 4F](#)). FMR1 expression level is linked to the degree of methylation of its 5'UTR sequence that could also impact the level of expression of miRNAs that are encoded nearby [23]. We then checked on TEC cultures if FMR1, miR-892b-3p and miR-20b-3p* were conjointly regulated by DNA methylation. TEC cultures were treated for 48h by 5-Azacytidine, a molecule causing hypomethylation of DNA. Changes in miR-20b-3p* expression could not be analyzed as this miRNA was hardly expressed in TECs. However, we observed a concomitant increased expression of FMR1 and of miR-892b-3p after demethylation ([Fig. 4G–H](#)). Altogether, these results

suggest that the degree of methylation of this chromosomal region could explain the low level of FMR1 as well as miRNAs, and could play a role in MG.

4. Discussion

MG patients with anti-AChR antibodies very often display thymic abnormalities with neoangiogenic processes and follicular hyperplasia characterized by the development of ectopic germinal centers. Here, we investigated dysregulated miRNAs that could help to understand the impact of post-transcriptional mechanisms on events leading to thymic changes associated with MG.

4.1. Specific dysregulated miRNAs in MG

We used two different approaches to search for dysregulated miRNAs in the MG thymus: a classical approach based on the fold change of miRNA expression and another approach taking into account the inverse correlation between the miRNA and mRNA expression levels. We validated by PCR the overexpression of miR-486-5p and miR-125a-5p, and the down-expression of miR-20b-3p*, miR-892b-3p and miR-7-5p. Among these miRNAs, we focus our interest on miR-7-5p as detailed below ([Section 4.2](#)), but miR-125a-5p and miR-20b-3p* are also of interest in the context of MG and autoimmunity. A decreased expression of miR-20b-3p* has already been observed in the serum of early-onset MG patients [24]. Xin et al. also showed a decreased expression of miR-20b in the serum of thymoma-associated MG, and in thymoma compared to non-thymomatous adjacent tissues. They demonstrated that the decrease of miR-20b could favor T-cell proliferation [25] but a lower expression level of miR-20b could also favor Th17-cell differentiation as observed in multiple sclerosis [26]. Lately, miR-20b has been characterized as an inhibitor of the inflammation by acting on NLRP3 (NLR Family Pyrin Domain Containing 3), a key protein of the inflammasome [27]. Therapies aiming at restoring miR-20b expression in MG but also in other autoimmune conditions could have a beneficial impact on disease evolution as described with Natalizumab in multiple sclerosis [28].

Here, we observed that miR-125a-5p was overexpressed in the thymus of early-onset MG patients and this miRNA has also been described as up-regulated in thymoma-associated MG. It is suggested that it could be involved in the regulation of FoxP3 expression which is decreased in thymoma-associated MG [29,30]. FoxP3 is an indispensable transcription factor for the generation and the regulatory function of regulatory T cells. The suppressive activity of regulatory T cells is severely impaired in early-onset MG patients and associated with a decreased expression of FoxP3 in CD4+ T cells [31]. Altogether, this suggests that miR-125a could play a role in the decreased functionality of regulatory T cells in MG patients. Here, we demonstrated an inverted correlation in the expression of miR-125a-5p and of WDR1, one of its mRNA target extracted with MAGIA2. WDR1 has been implicated in auto-inflammatory processes associated with IL-18 expression [22]. This observation is of particular interest as IL-18 is overexpressed in the serum of MG patients [32] and the administration of an anti-IL-18 antibody to myasthenic rats leads to the alleviation of clinical symptoms [33]. miR-125a could also target other mRNAs such as TNFAIP3 (Tumor Necrosis Factor Alpha-Induced Protein 3), also known as A20. TNFAIP3 is essential for the development and function of immune cells and has been implicated in the pathogenesis of various autoimmune diseases [34]. TNFAIP3 is a key molecule in the negative regulation of NF- κ B (Nuclear Factor κ B)

Fig. 3. Inverse correlation for miRNA and mRNA expression data Inversely correlated miRNA-mRNA interactions were analyzed with MAGIA2. Networks of miRNAs (orange triangles), mRNAs for transcription factors (green circles) and mRNAs for other genes (blue circles) were built using the prediction databases Targetscan (A) or DIANA micro-T (B). The miRNAs and their targets are listed in [Table 3](#). RT-PCR data for miR-125a-5p (C), WDR1 mRNA (D) in the thymus of controls (n = 6) and MG patients (n = 12). mRNA and miRNA expression levels were given in arbitrary units and were normalized to 28S. p-Values were assessed by the Mann-Whitney test and only p-values < 0.05 are indicated (*p < 0.05). (E) Inverse correlation for miR-125a-5p and WDR1 mRNA expression in the thymus of controls (n = 6) and MG patients (n = 12). Pearson's correlation was assessed and p-values under 0.05 are indicated.

Table 3

Dysregulated miRNAs and their targets, according to TargetScan and DIANA-microT.

MAGIA2 used the TargetScan and DIANA micro-T databases to associate dysregulated genes in MG thymus and putative miRNA targets. Dysregulated miRNAs were extracted from TAC analysis whereas dysregulated genes in the thymus were extracted from a previous transcriptomic study as fully detailed in the [Materials and methods section](#).

Name	FC (MG/Control)	p-Value	TargetScan	DIANA MicroT	Targets in dysregulated gene in the MG thymus (EnterGene ID)
hsa-miR-486-5p	3.18	0.009		X	DIDO1, DST, GSN, MKRN1, PPP2R5D, STT3B
hsa-miR-574-3p	2.15	0.001		X	DIDO1, DNMI
hsa-miR-612-5p	1.64	0.028		X	ATP6V1D, DIDO1, DST, FAM155B, GPANK1, HPCAL1, LTN1, PFDN1, TCL1A, TRIM29
hsa-miR-760-3p	1.56	0.032		X	CNOT4, CTSC, EPB41L3, FAM155B, HLA-DQB1, GSN, IGF2R, KRT8, LMNB2, TTN
hsa-miR-125a-5p	1.45	0.016	X	X	CARS2, DIDO1, DST, DUST7, ENTPD4, FAM155B, HDGF, MCL1, RAB2B, RFX5, SMARCD2, WDR1
hsa-miR-4465-3p	-1.19	0.050	X		ALDH5A1, ERP44
hsa-miR-875-3p	-1.22	0.039		X	DIDO1, DST, EDNRA, HBS1L, HDGF, HPCAL1, LOM7, RAB2B, TTN, ZMYM4
hsa-miR-767-3p	-1.36	0.004		X	ANGEL1, ALDH5A1, DST, MAPKAPK3, MYD88, NCKAP1L, PFDN1, SPG7, SPOP, TTN
hsa-miR-891b-5p	-1.49	0.006		X	AUTS2, CD1C, DST, HBS1L, HNRNPC, NSF, PPP1CC, SPG7, TAF11, ZFY
hsa-miR-551a-3p	-1.54	0.017		X	HSF1, LMO7, TAPBP,
hsa-miR-421-3p	-1.85	0.041		X	DST, EPB41L3, HBS1L, HPCAL1, IDH1, MAPKAPK3, RAB2B, SPG7, TAF15, ZMYM4
hsa-miR-1-3p	-1.86	0.000	X	X	C16orf62, FOSB, FAM155B, MKRN1, MS4A1, NSF, PFDN1, SDCBP, UTRN, WDR1, ZNF292
hsa-miR-34c-5p	-1.91	0.050	X	X	ANKRD12, BCL6, EPB41L3, FAM155B, HBS1L, IDH1, LMAN1, PPP2R5D, TRIM29, ZMYM4
hsa-miR-4500-3p	-1.98	0.011	X		ANKRD12, ATP2B4, CCL3, DUSP7, DZIP1, FASLG, KLF9, LTN1, PLEKHO1, UTRN
hsa-miR-892a-3p	-1.99	0.007		X	ALDH5A1, ANKRD12, DIDO1, DST, HDGF, KLF9, LAMC1, PEBP1
hsa-miR-375-3p	-2.27	0.045		X	B4GALT5, DST, EPB41L2, EIF4H, LAMC1, MAN1A2, SCN3A, SPOP, ZFP36L2
hsa-miR-509-3p	-2.27	0.008		X	DST, ERP44, RFX5
hsa-miR-890-5p	-2.66	0.020		X	BTG1, DIDO1, DST, DZIP1, EPB41L2, EPB41L3, HDGF, MAN1A2, UTRN, ZMYND11
hsa-miR-429-3p	-3.37	0.031	X	X	DIDO1, DNAJB5, DST, DZIP1, IDH1, IGF2R, LAMC1, LMO7, LTN1, MKRN1, MTMR14, NPC1, SSR3, ZNF292

and also TLR (Toll-Like Receptor) signaling pathways [35,36]. Consequently, increased expression of miR-20b and miR-125a could modulate inflammatory pathways and could play a key role in MG and in other autoimmune diseases [34,37].

Consequently, these observations suggest that miRNAs such as or miR-125a-5p can modulate inflammatory pathways and could play a key role in MG and in other autoimmune diseases.

4.2. miR-7 downregulation is involved in thymic changes

We validated that miR-7-5p was the most down-regulated miRNA in MG thymuses and this was due to its down-regulation in TECs. We showed that the level of miR-7 expression was even more decreased in highly hyperplastic thymuses. Interestingly, in corticoid-treated patients, miR-7 expression was higher than in hyperplastic thymuses. Altogether, this suggests that miR-7 could vary along with treatments and the hyperplastic state of the thymus.

miR-7 was initially described in cancers but more recently its potential implication in autoimmune conditions has been highlighted. miR-7 expression is downregulated in the serum of patients with dermatomyositis and neuromyelitis optica [38] [39]. However, we did not observe such decrease in the serum of MG patients (data not shown). miR-7 expression seems to be decreased in inflammatory tissues. As observed in MG thymuses, miR-7 is also downregulated in the skin of dermatomyositis patients [38] and in actively inflamed Crohn's disease colonic tissues [40].

Using prediction databases, we found that miR-7-5p can interact with the 3'-UTR region of CCL21. More, we observed that miR-7-5p and CCL21 mRNA expression were inversely correlated in the thymus of MG patients. CCL21 plays an important role in thymopoiesis. It is mainly secreted by TECs promoting the recruitment of prothymocytes and later on the migration of CD4+ thymocytes towards the medulla region [41]. In the MG thymus, CCL21 is abnormally overexpressed and this is mainly due to ectopic lymphatic vessels [4], but also to a lesser extent by TECs. Indeed, IFN- β that is overexpressed in MG thymuses can induce CCL21 expression in lymphatic endothelial cells and TECs [5]. As miR-7-5p down-regulation was observed in MG TECs, we investigated its effects by transfecting miR-7-5p in primary TEC cultures. We showed that miR-7-5p could decrease CCL21 release by TECs. This suggests that decreased miR-7-5p expression in MG thymuses could affect CCL21 expression and disturb intrathymic-cell

migration. We did not investigate the impact of miR-7-5p transfection in thymic lymphatic endothelial cells that have been shown to highly expressed CCL21 in MG thymuses. The level of post-transcriptional regulation of mRNAs by miRNAs is probably cell-dependent and the effects of miR-7-5p could be higher in lymphatic endothelial cells.

Altogether our results suggest that an abnormal expression of miR-7 could participate in the abnormal expression of CCL21 associated with thymic changes observed in MG patients.

4.3. Dysregulated miRNA clusters suggest the implication of X chromosome in MG

Analyzing the chromosomal localization of dysregulated miRNAs, we observed a significant overrepresentation of two chromosomal regions. miRNAs overexpressed in 14q32.33 correspond to a cluster of miRNAs, including miR-4537, miR-4538, miR-4539 and miR-4507. This chromosomal region was also found significantly overrepresented in a thymic transcriptome study of MG patients [18]. It corresponds to the immunoglobulin heavy chain locus and its overrepresentation likely reflects the increased number of B cells in MG thymus. The miRNAs encoded by this cluster are not considered as intragenic miRNAs but they could be transcribed together with immunoglobulin heavy chain genes as they are encoded on the same DNA strand and on overlapping regions.

Analyzing specific dysregulated miRNA from Table 2A, we also found a significant overrepresentation of down-regulated miRNAs encoded by the Xq27.3 and Xq26.2 regions. The importance of miRNAs in the Xq27.3 region was also pointed out by our analysis of inverse correlation for miRNA and mRNA expression (Table 3). The dysregulated miRNAs in these chromosomal regions has not yet been linked to autoimmunity and inflammation, except miR-20b, as detailed in Section 4.1. As many autoimmune diseases, MG is predominantly observed in females, and autoimmunity is much higher in patients with X-linked disorders [42]. The Xq27.3 chromosomal region encodes for the FMR1 gene implicated in the Fragile X syndrome, a form of X-linked mental retardation. This syndrome results from the expansion of CGG repeat sequences in the 5' untranslated region of the FMR1 gene. Normal alleles are considered to have between 5 and 44 CGG repeats, alleles with 45 to 54 repeats are intermediate (grey zone), alleles with 55 to 200 repeats constitute premutated FMR1 and over 200 repeats alleles present a full mutation. Individuals with FMR1 full mutation or premutations

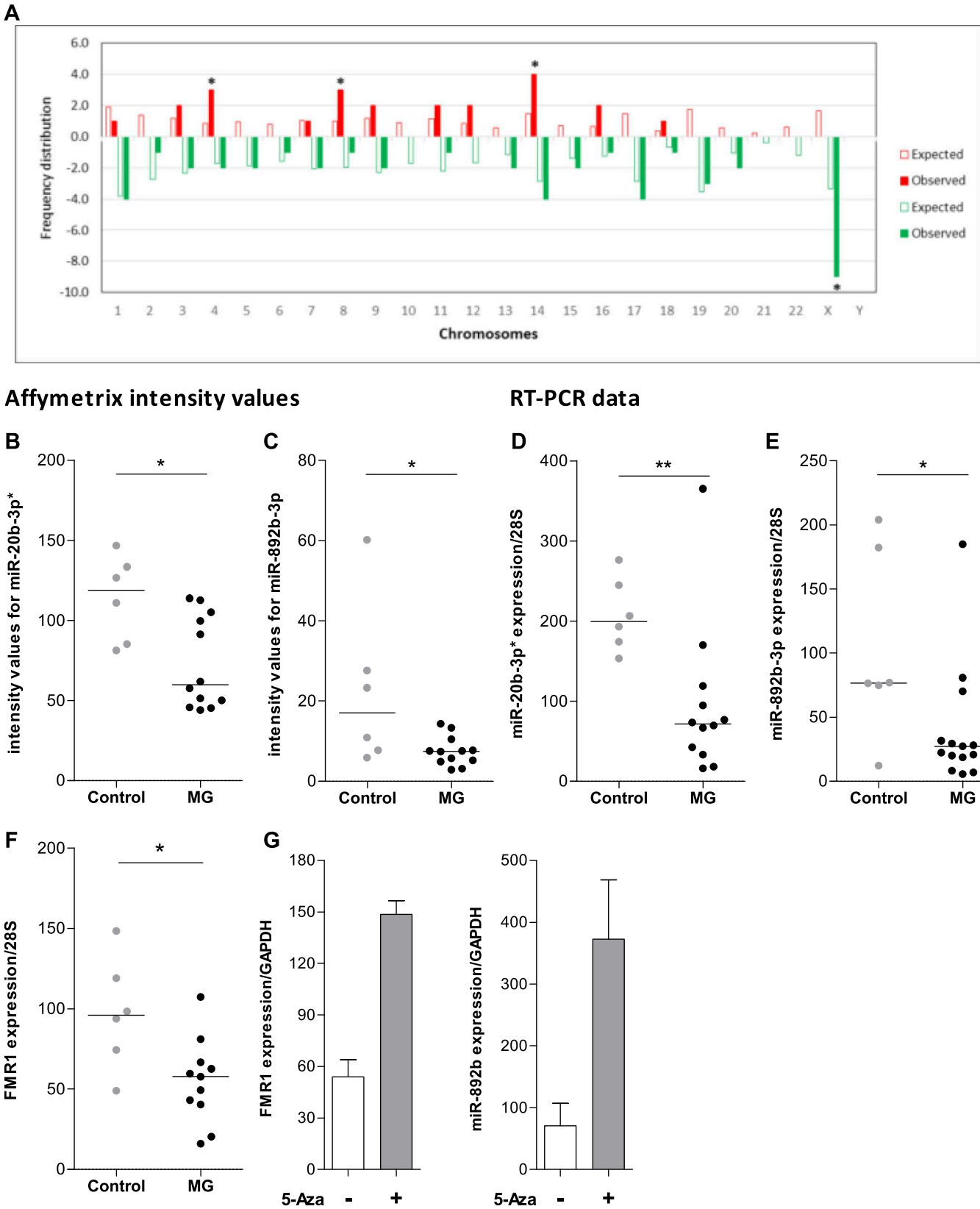


Fig. 4. Expression of miRNAs and FMR1 mRNA located on the same region of the X chromosome. (A) The bar chart indicates the expected frequency of miRNAs on each chromosome compared to the observed frequency extracted from the list of significantly overexpressed (in red) or down-regulated (in green) mature miRNAs listed in Table 2A. If the p-value for the calculated Chi-square test was <0.05 (*), the hypothesis was rejected, meaning that the proportion of dysregulated miRNAs on a given chromosome is significantly higher than expected. Intensity of fluorescence of miR-20b-3p* (B) and miR-892b-3p (C) from the Affymetrix chips for non-MG adults (controls, $n = 6$) and MG patients ($n = 12$). RT-PCR data for miR-20b-3p* (D), miR-892b-3p (E) and FMR1 mRNA (F) expression in the thymus of controls ($n = 6$) and MG patients ($n = 11$). mRNA expression levels were given in arbitrary units and were normalized to 28S expression. Representative experiment of FMR1 (G) and miR-892b-3p (H) expression in TECs treated with 5-Azacytidine ($5 \mu\text{M}$) for 48 h. mRNA and miRNA expression levels were given in arbitrary units and were normalized to GAPDH. P-values were assessed by the Mann-Whitney test and only p-values < 0.05 are indicated (* $p < 0.05$, ** $p < 0.01$).

display immune-mediated disorders and in particular a higher risk to develop autoimmune diseases [43,44].

The regulation of FMR1 expression is very complex and dependent on the number of CGG repeat sequences in 5'UTR and the subsequent DNA methylation level, especially for individuals with the full mutation genotype. However, the presence of a methylation boundary region upstream FMR1 promoter and of four CTCF binding sites is also important for FMR1 expression. CTCF is an important transcriptional regulator of genes harboring trinucleotide repeats and it is mostly active in chromatin organization [45,46].

Analyzing FMR1 mRNA expression, we observed a significant decreased expression in the thymus of MG patients. Of note, the expression of FMR2/AFF2, a gene localized near FMR1 was also strongly decreased in the thymus of MG patients (data not shown). We also demonstrated that demethylation with 5-Azacytidine of control TEC cultures increased FMR1 expression concomitantly with the increased expression of miR-892b-3p. miRNAs that were found down-regulated in our study could have been impacted by the higher methylation status of DNA in this region even if there are on opposite strands [47]. This suggests that genes and miRNAs encoded in this chromosomal region could be conjointly regulated and it raises the issue of a link between autoimmunity and this chromosomal region.

5. Conclusion

Studies have been carried out to investigate the implication of miRNAs in physiological and pathophysiological mechanisms. Here for the first time, we investigated with different approaches dysregulated miRNAs in the thymus of MG patients. We demonstrated that specific miRNAs were implicated in thymic changes associated with MG, in particular, miR-7-5p but others such as miR-125a-5p and miR-20b-3p* are also of interest. We also demonstrated a decreased expression of genes and miRNA clusters localized at the extremity of the X chromosome with a potential link with autoimmunity. Altogether this detailed analysis of the thymic miRnome is of great interest bringing new knowledge in the etiological and pathophysiological mechanisms involved in early-onset MG.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.autrev.2018.01.008>.

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Authorship

MAC, SM, FD, NS performed and analyzed the experiments, FT collected samples and provided patient information, MF performed the miRnome experiment, EF and JG provided thymic biopsies, SB-A and SM read and revised the manuscript, MAC and RLP designed the study, analyzed the experiments and wrote the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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ARTICLE 2

Involvement of miR-29s in the pathogenesis of autoimmune Myasthenia Gravis

Article in preparation

Objectives

Early-onset Myasthenia Gravis (MG) patients often display thymic abnormalities such as ectopic B-cell infiltrates organized in germinal centers. Our team demonstrated that dsRNA, sign of an infection mimicked by Poly(I:C), induces the release of IFN- β and specifically induces the expression of α -AChR in thymic epithelial cells. Poly(I:C) treatment was also able to induce the overexpression of CXCL13 and CCL21, two chemokines involved in thymic hyperplasia. All these signals were abolished upon the blockade of IFN-I signaling (*in vitro* and *in vivo* with IFNAR KO mice), suggesting a strong role of IFN-I in the pathogenesis of MG. [260, 261]. In the meantime, the team of Dr A. Liston demonstrated that the absence of miR-29a in the thymus led to a higher sensitivity to pathogen signals through its effects on *Ifnar1*, the IFN-I receptor, in mice.

Knowing the implication of the IFN-I pathway in the pathogenesis of MG, the objective of this study was to evaluate the importance of the miR-29 family in MG. [308].

For that, we investigated the expression profile of the miR-29 family in the thymus of MG patients. We showed that the whole miR-29 family was down-regulated in MG thymuses. We also showed that miR-29a KO mice were more sensitive to the experimental model for MG, which was consistent with the conclusions of Papadopoulou *et al.* regarding the increased sensitivity to pathogen signals through *Ifnar1* up-regulation.

Additional investigations are ongoing and experiments will be conducted in the near future regarding other miR-29a targets.

This study is willingly presented with preliminary results formatted into an article in order to be homogenous with other results presented in this manuscript.

Involvement of miR-29s in the pathogenesis of autoimmune Myasthenia Gravis

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Abbreviations:	CFA:	complete Freund's adjuvant
	HET:	heterozygous
	IFN:	interferon
	KO:	knock-out
	MG:	Myasthenia Gravis
	miRNA:	microRNA
	TEC:	thymic epithelial cell
	WT:	wild-type

ABSTRACT

Myasthenia Gravis (MG) is a rare autoimmune disease mediated by the autoantibody attack of the acetylcholine receptor (AChR) at the neuromuscular junction. The thymus is the effector organ of the disease as it displays functional and morphological abnormalities leading to follicular hyperplasia and production of AChR antibodies. However, as in many autoimmune disorders, MG etiology is not clearly defined. It was demonstrated that type-I interferon (IFN-I) and especially IFN- β could act as the orchestrator of thymic abnormalities observed in MG. The miR-29a/b-1 cluster has been described as a regulator of *Ifnar1*, the IFN-I receptor in mice, and is responsible for the higher sensitivity to IFN-I signals. The objective of this study was to investigate the expression of miR-29 miRNAs in the thymus of MG patients and to evaluate the sensitivity of miR-29a/b-1-deleted mice to the EAMG model.

We showed that all miR-29 miRNAs were down-regulated in the thymus of MG patients, as a family or individually. Moreover, we demonstrated that miR-29 HET and KO mice were more sensitive to the EAMG model compared to CFA-injected and WT-T-AChR injected mice.

Further investigations are ongoing to determine the mechanisms involved in the increased sensibility of the EAMG model of these mice.

INTRODUCTION

MicroRNAs (miRNAs) are potent modulators of protein expression and are therefore involved in many physiological and pathophysiological processes. We previously demonstrated that miRNAs could be involved in the pathogenesis of an autoimmune disease, Myasthenia Gravis (MG) (Cron et al, 2018). MG is a rare disease characterized by more or less invalidating muscle weaknesses, due to auto antibodies directed against components of the neuromuscular junction, such as the acetylcholine receptor (AChR). In early-onset MG with anti-AChR antibodies, if the target organ is the muscle, the effector organ is the thymus. The thymus is often characterized by active neoangiogenic processes, with the development of high endothelial venules and lymphatic vessels and the overexpression of chemokines, such as CXCL13 and CCL21 that allow the recruitment of peripheral cells (Berrih-Aknin et al, 2009; Meraouna et al, 2006; Weiss et al, 2013). Indeed, the MG thymus is characterized by abnormal B-cell infiltrations leading to ectopic germinal center development (thymic follicular hyperplasia) involved in the production of anti-AChR antibodies (Truffault et al, 2017).

The thymus is a common target organ for infectious diseases (Savino, 2006) and an anti-viral signature has been observed in MG thymuses (Le Panse et al, 2006). Moreover, poliovirus-infected macrophages and Epstein-Barr virus (EBV)-infected B cells have been described in the thymus of MG patients (Cavalcante et al, 2010a; Cavalcante et al, 2010b). In MG patients, thymic overexpression of IFN- β is also observed even long after disease onset (Cufi et al, 2013). In addition, polyinosinic-polycydidylic acid (Poly(I:C)) mimicking dsRNA from viral infections can induce an anti-AChR autoimmune response in mice. It was demonstrated that IFN- β , released upon dsRNA signaling activation, can induce thymic changes that could initiate the anti-AChR response (Cufi et al, 2013). Next, Cufi *et al.* further demonstrated that IFN- β is the orchestrator of thymic molecular changes that could lead to AChR sensitization and chemokine expression and consequently peripheral cell recruitment, germinal center development and the emergence of auto-reactive B cells (Cufi et al, 2014).

A few years ago, Papadopoulou *et al.* demonstrated that miRNAs are essential to the thymic architecture and further demonstrated that miR-29a leads to an increased sensitivity to pathogens (Papadopoulou et al, 2012). In this study, the authors first investigated the involvement of miRNAs in the rapid thymic involution after a pathogen exposure. For that, they performed a conditional knock-out (KO) of *Dicer*, a major protein of the biogenesis of miRNAs, in thymic epithelial cells and demonstrated that *Dicer* and miRNAs were essential to thymic architecture. Indeed, upon *Dicer* deletion, they observed epithelial voids causing a loss of cellularity, premature thymic involution and dense B-cell foci. They next investigated the involvement of miR-29a, as it targets *Ifnar1* mRNA, the type-I interferon (IFN-I) receptor and the most upregulated member of the IFN-I signaling pathway upon *Dicer* deletion. They showed that miR-29 KO mice displayed a higher sensitivity to pathogen signals due to the higher threshold set by up-regulated IFNAR1. Another study from the same group demonstrated that the miR-29a/b-1 cluster conferred protection against induced collagen arthritis because of a B-cell defect after stimulation. In addition, the authors identified the miR-29a/b-1 cluster as an essential component of immune response and suppressor of inflammatory cytokines, compared to miR-29-b-2/c (van Nieuwenhuijze et al, 2017).

In this study, we investigated the expression of the miR-29 family in the thymus of MG patients. According to a miRnome study previously published (Cron et al, 2018), we observed and further validated by RT-PCR that all the members of the miR-29 family were down-regulated in the thymus of MG patients. Then, we investigated the sensitivity of miR-29a/b-1-deleted mice to the EAMG model. We showed that miR-29a/b-1-deleted mice developed stronger MG symptoms, suggesting that the miR-29 miRNAs may have a role in the etiology and/or pathogenesis.

MATERIALS AND METHODS

Thymic samples

For the miRnome analysis, patients and controls were as previously described (Cron et al, 2018). For qPCR analysis, thymic biopsies from early-onset MG patients (n=12, 15-35 years old) were collected after thymectomy and control thymic biopsies (n=6, 15-33 years old) were collected from donors undergoing cardiovascular surgery, both at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France). MG patients were only treated with cholinesterase inhibitors and had no other known diseases including thymoma; all their characteristics are detailed in Table 1. All the studies on thymuses were approved by a local ethics committee (CPP, authorization number ID RCB 2010-A00250-39) and informed consent forms have been collected.

Animals

Thymic miR-29a/b-1 (miR-29)-deleted mice were kindly given by Dr A. Liston and designed as previously described (Papadopoulou et al, 2012). C57BL/6j mice were purchased at Janvier Labs (Saint-Berthevin, France). For this project, all mice were bred and kept in a specific pathogen free animal care facility (Centre d'Expérimentation Fonctionnelle, Sorbonne Université, Paris, France) according to European and French ethics agreements (n° 2569.01).

Experimental autoimmune Myasthenia Gravis (EAMG)

To perform experiments with enough animals, we used 6-8 week-old C57BL/6 male and female mice, either inbred miR-29 KO, HET or WT mice on the C57BL/6 background or C57BL/6j from Janvier Labs, as a comparison to previous experiments carried out in the laboratory (Robinet et al, 2017; Weiss et al, 2016). It is worth mentioning that no differences are observable in the EAMG model between male and female (Tuzun et al, 2015).

Extraction of Torpedo Californica AChR (TACHR) was led as previously described (Robinet et al, 2017). T-AChR was emulsified with an equal volume of Complete Freund's adjuvant (CFA; #F5881, Life Technologies, Villebon-sur-Yvette, France) supplemented with heat-inactivated *Mycobacterium tuberculosis* (10 mg/ml, #H37RA, BD Difco, Villepinte, France). Mice were subcutaneously injected (200 µl/mouse, 30 µg AChR) at several sites (hind foot-pads, tail base and in the back). Control mice were injected similarly with CFA emulsion devoid of T-AChR. After four weeks, mice were immunized a second time with T-AChR emulsified in CFA only at the tail base and in the back.

The global clinical score was noted from 0 to 9 by taking into account the weight loss, the muscle strength and the inverted grid test. Each of these measures was graded on 3 as fully described by Weiss *et al.* (Weiss et al, 2016). The grip test measurements were done after a three-minutes run in a treadmill. For the weight and grip test analyses, the value obtained was reported to previous values obtained prior to the experiments and converted as a percentage. This percentage, representing the loss/gain of weight and muscle strength, was attributed to a clinical score, as detailed by Weiss *et al.* (Weiss et al, 2016). Grip test values were normalized on the weight of the animal, since experiments were performed on both female and male mice. For the inverted grid test, mice were first tired by gently dragging them across a grid 20 times. Immediately after, the grid was inverted and held steadily for 1'30". During this time lapse, mice were carefully observed to detect any sign of abnormal behavior. Of notice, a mouse was considered sick when it reached a global clinical score of 2. A mice reaching a global clinical score of 9 was considered too sick and was euthanized.

RNA extraction

Total RNA was extracted from thymic biopsies using the mirVana miRNA Isolation Kit. Biopsies were first lysed in the Lysis/Binding buffer provided in the mirVana kit using the FastPrep FP120 instrument (Qbiogen, Illkirch, France). RNA integrity was assessed on a Bioanalyzer 2100 (Agilent Technologies, Les Ulis, France).

Retro-transcription and polymerase chain reaction (RT-PCR) for miRNAs

miRNAs were retro-transcribed from total RNA using the TaqMan MicroRNA Reverse Transcription Kit and following the Custom RT Pool protocol (Life Technologies, Villebon-sur-Yvette, France). Briefly, up to seven RT primers were pooled with RNA samples so that the miRNAs expression could be assessed in a unique sample. qPCR reactions were carried out using the TaqMan Universal Master Mix II, no UNG (Life Technologies) on a LightCycler 480 (Roche, Meylan, France). PCR settings were as followed: 1 cycle of polymerase activation and denaturation at 95°C for 10 minutes, 45 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute. Primers used for qPCR are listed on Table 2.

ELISA

ELISA experiments were carried out on serum samples collected at sacrifice (day 43). 96-well plates were coated with 0.5 µg/mL of TACHR diluted in 10 mM NaHCO₃ buffer, pH 9.6, overnight at 4°C. Wells were blocked with PBS 10% fetal calf serum for 2h30 at 37°C. Next, samples diluted in PBS 0.2% BSA (1:100 000) are incubated at 37°C for 1h30. 100 µL of 1/10 000 diluted biotinylated rabbit anti-mouse IgG (#E0413, Dako, Courtaboeuf, France) were added for 1h30 at 37°C. Next, samples were incubated with 100 µL of streptavidin–horseradish peroxidase (diluted at 1:10 000) (#S911, Thermo-Fisher Scientific, Villebon-sur-Yvette, France). Tetramethylbenzidine was used for color development, and the optical density at 450 nm was measured with the SPARK 10M microplate reader (TECAN Life Sciences, Grödig, Austria). Between each step, wells were washed 4 times with 200µl of PBS 0.05% Tween 20.

Statistical analysis

For 2-by-2 comparisons, the non-parametric Mann–Whitney test was applied as specified in figure legends.

RESULTS

The miR-29 family is down-regulated in the thymus of MG patients

Using miRnome data previously published (Cron et al, 2018), we observed a significant decrease of all the members of the miR-29 family (miR-29a-3p, miR-29c-3p, miR-29b2-5p, miR-29b-3p, miR-29c-5p, miR-29b1-5p and miR-29a-5p) (Figure 1A). We can notice that in human, miR-29 miRNAs are transcribed from two different chromosomes: the miR-29a/b-1 cluster is encoded on chromosome 7 while the miR-29c/b-2 cluster is encoded on chromosome 1. In mice, both clusters are encoded on chromosome 1. To validate the miRnome observations, we analyzed by RT-PCR the expression of all seven miRNAs. We showed again that altogether, miR-29 miRNAs were all significantly down-regulated in MG thymuses compared to controls. In addition, we demonstrated that at the individual level, miR-29 miRNAs were all significantly down-regulated in MG thymuses, except the very low expressed miR-29b-1-5p (Figure 1B). As described for the mouse thymus (Papadopoulou et al, 2012), we confirmed that miR-29a-3p was the most strongly expressed miRNA and this was especially obvious with the microarray analysis.

miR-29a/b-1-deleted mice display higher sensitivity to MG

We investigated the sensitivity of miR-29 KO, HET and WT mice to MG. We first had troubles generating a correct number of mice as they did not well reproduced. This issue led us to mix males and females for our experiments and to add C57BL/6 mice from Janvier Labs as control groups for CFA.

First, we showed that C57BL/6 WT and miR-29 WT mice injected with T-AChR were similarly sensitive to the EAMG model (Figure 2A). Next, we demonstrated that miR-29 HET and KO mice were more sensitive to the EAMG model compared to WT “Janvier” mice, as they displayed a higher global clinical score when HET and KO mice were pooled. If the miR-29 WT and the HET+KO groups were not significantly different, it may be due to the small number of mice in the WT group. However, compared to CFA-injected C57BL/6 mice, miR-29 HET and KO TACHR-injected mice displayed a significant

increased global clinical score (Figure 2A). Interestingly, we observed a “genotype” effect, where KO mice are more severely affected than HET mice (100% vs. 84.6%).

We then measured the anti-T-AChR antibody titers and showed that all mice immunized with T-AChR developed anti-AChR antibodies that were not detected in control CFA-injected mice. However, no significant differences were observed in C57BL/6 WT and miR-29 WT, HET and KO mice (Figure 2B). The discrepancies between the symptom improvement and the constant level of anti-AChR antibodies can be explained by the fact that the antibody titer does not correlate with the severity of the disease in human (Gilhus, 2016).

Ongoing analyses

To confirm the higher susceptibility of miR-29 HET and KO mice to EAMG, we will measure the AChR density on diaphragm muscles using radioimmunoassay (RIA). As the antibody level is significantly higher in miR-29 HET and KO mice compared to CFA-injected mice, we expect to see fewer AChR on the diaphragm muscle of these mice. We will also investigate the neuromuscular junction structure in muscles of miR-29 mice to see if muscle weaknesses could result from neuromuscular junction disorganization.

In the EAMG model, mice do not display thymic changes, such as follicular hyperplasia often observed in the human pathology. As miR-29 HET and KO mice display a higher sensitivity to IFN-I that could lead to thymic changes, we will analyze by immunohistochemistry the thymus of miR-29 WT, HET and KO mice in the EAMG model searching for B-cell infiltration and ectopic follicle development.

DISCUSSION

This study aimed to investigate the potential implication of miRNAs from the miR-29 family in MG, whether in the thymus of MG patients or in the EAMG mouse model.

In the context of MG, the miR-29 family is very interesting and in particular miR-29a. Indeed, in a study published in 2012, the importance of miRNAs in thymic architecture was showed by deleting *Dicer* specifically in mouse thymic epithelial cells (TECs). They observed, along with the growth and aging of the animals, premature thymic involution and formation of epithelial voids in the cortex and in the medulla suggesting a role for miRNAs in the prevention of apoptosis in thymuses. Interestingly, they observed the presence of dense B-cell foci in *Dicer*-deleted thymuses voids.

Next, as it was demonstrated that thymic changes in mice deleted for *Dicer* in TECs were mainly mediated by the cluster miR-29a/b-1. The authors showed that upon deletion, *Ifnar1* is upregulated and that miR-29 KO mice are more sensitive to pathogen signals demonstrated with the injection of Poly(I:C), a double-strand RNA mimicking viral infection (Papadopoulou et al, 2012).

Knowing the role of type-I interferon (IFN-I) and especially IFN- β as orchestrator of thymic abnormalities in MG (Cufi et al, 2014), and that Poly(I:C)-injected mice can develop MG symptoms associated with anti-AChR antibodies (Cufi et al, 2013), we analyzed the role of miR-29 miRNAs in MG. Surprisingly, in the MG thymus, we also initially observed a decreased expression of *DICER* in the thymus of MG patients compared to non-MG controls (data not shown). In parallel, we investigated the expression level of the miR-29 miRNAs in the thymus of MG patients and showed that all the miR-29 miRNAs were down-regulated. This observation suggests, as in Papadopoulou *et al.*, that miR-29a/b-1 deletion or down-regulation could increase the sensibility to IFN-I, and consequently participate to MG. However, we did not observe any variation in the expression of *IFNAR1* in the thymus of MG patients (data not shown). Moreover, *Ifnar1* is not listed as targeted by miR-29a according to the usual sequence matching databases (miRanda, TargetScan and DIANA microT) in human but not in mouse as well, while Papadopoulou *et al.* clearly describe *Ifnar1* expression in the

thymus of miR-29 KO mice (Papadopoulou et al, 2012). miR-29a/b-1 could be involved in the increased susceptibility to develop MG but via an alternative IFN-I pathway. For example, in multiple sclerosis, miR-29a/b-1 cluster is thought to be a repressor of T_H1 cells by acting on T-bet and IFN- γ (Smith et al, 2012). It was also characterized as an IFN- γ repressor in the context of a bacterial infection (Ma et al, 2011). In MG, we observed an overexpression of IFN- γ (Gradolatto et al, 2012) and the down-regulation of the miR-29 family, reinforcing the involvement of the miR-29 family in the pathogenesis of MG.

Interestingly, miR-29 members are known to target TNF-alpha induced protein 3 (TNFAIP3, also known as A20) (Balkhi et al, 2013). TNFAIP3 pathway is important in MG because a single nucleotide polymorphism (SNP) in *TNFAIP3* sequence is associated with late-onset MG (Yang et al, 2017), as well as a SNP in TNIP1 (TNFAIP3 interacting protein 1) with early-onset MG (Gregersen et al, 2012). No studies were led regarding the expression of *TNIP1* or *TNFAIP3* in the thymus of MG patients, only in preliminary data in which Wang & Gung saw a down-regulation of *TNIP1* in MG thymocytes (Wang & Gung, 2015). It would be interesting to look at the expression of these genes in MG thymuses and TECs to make a link between the down-regulation of the miR-29 family in MG thymuses and *TNIP1/TNFAIP3* gene expression. However, TNFAIP3 role is characterized as anti-inflammatory as it inhibits NF- κ B signaling pathway (Boone et al, 2004) and its implication in MG remains to be elucidated, where inflammation is uncontrolled.

Next, we demonstrated that miR-29 HET and KO mice displayed a higher sensitivity to EAMG according to the global clinical score but did not display a higher anti-T-AChR antibody titer. The severity of the disease is not correlated with the antibody titer (Gilhus et al, 2016) but is correlated with the degree of hyperplasia (Truffault et al, 2017). In the EAMG model, mice do not display thymic abnormalities. However, knowing that *Dicer* deletion, probably through the down-regulation of miR-29a, causes the emergence of dense B-cell foci comparable to germinal centers, it would be interesting to look at B cells and possible ectopic lymphoid follicles in the thymus of miR-29 mice.

In contrast to our observations in the EAMG model, another study demonstrated that the deletion of the miR-29a/b-1 cluster conferred near-complete protection from collagen-induced arthritis, with profound defects in B-cell proliferation and antibody production. Investigating the underlying protective mechanisms, they demonstrated that miR-29a has a critical function in the B-cell germinal center response (van Nieuwenhuijze et al, 2017). Thus, miR-29a/b-1 could favor the development of B-cell dependent autoimmune diseases. Smith *et al.* also demonstrated that the deletion of the miR-29a/b-1 cluster protects from the development of multiple sclerosis in mice by inducing CD4⁺ T-cell apoptosis in draining lymph nodes and a decreased cellular infiltration in the spinal cord (Smith et al, 2012). However, they showed that miR-29a/b-1 deletion led to a more pronounced T_H1 phenotype, resulting in a higher IFN- γ production and *T-bet* expression. These observations can be transposed to what we see in MG, *i.e.* an increased production of T_H1-related cytokines (Villegas et al, 2018) that could explain the higher sensitivity of miR-29a/b-1 HET and KO mice to EAMG. In addition, Papadopoulou *et al.* suggested that the decreased cellularity observed in the thymus of miR-29 HET and KO mice could lead to a less efficient positive selection, further leading to auto-reactive T cell production (Papadopoulou et al, 2012).

Altogether, these results suggest that the miR-29a/b-1 cluster is crucial in the establishment of a controlled immune system. In addition, miR-29a/b-1 could be anti-pathogenic in T-cell dependent diseases and pro-pathogenic in a B-cell dependent disease, as suggested by van Nieuwenhuijze *et al.* (van Nieuwenhuijze et al, 2017), explaining the different phenotypes observed in autoimmune mouse models.

CONCLUSION

The objective of this study was to investigate the implication of the miR-29 family in an autoimmune context, knowing its involvement in the IFN-I pathway. We showed that all 7 members of this family were down-regulated in the thymus of MG patients. We also demonstrated that miR-29a/b-1 mutated (heterozygous and knock-out) mice displayed higher sensitivity to the EAMG model. However, it is difficult to conclude about the pathway involved in this increased sensibility. The hypothesis involving *Ifnar1* in the elevated threshold regarding pathogen sensitivity is valid but we were not able to demonstrate an overexpression of *IFNAR1* in MG thymuses and in miR-29a/b-1 HET and KO mice. Further investigations are needed to highlight involved pathways and how could they contribute to MG pathogenesis, such as experiments using Poly(I:C) in miR-29 KO mice.

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The authors do not declare any conflict of interest.

Figure legends

Figure 1: miR-29 family is down-regulated in human MG thymuses

(A) miR-29 family expression was assessed by microarray in MG thymuses (n=6) compared to non-MG female control thymuses (n=6). Each point corresponds to the mean of intensities for the six values for non-MG controls and MG thymuses.

(B) miR-29 family expression was assessed by RT-PCR in MG thymuses (n=6) compared to non-MG female control thymuses (n=6).

For each individual miRNA, p-values were assessed by the Mann-Whitney test and only those over 0.05 are indicated next to the miRNA name. For the comparison of all miR-29 miRNAs together, p-value was assessed by the Wilcoxon test and are indicated on top of the graph.

Figure 2: miR-29 mice are more sensitive to the EAMG model

C57BL/6, wild-type, heterozygous and knock-out miR-29 mice were immunized at day 0 and boosted at day 29 with complete Freund's adjuvant (CFA) or CFA/Torpedo californica AChR (T-AChR). Clinical evaluations were done at days 14, 27 and 43, and data obtained at day 43 are shown on graphs. Male mice are represented as empty black dots.

(A) A global clinical score was calculated for each mouse taking into account the weight loss, the grip test, and the inverted grid test, as detailed in the method section. (B) ELISA for anti-T-AChR antibodies in serum at day 43.

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Figure 1

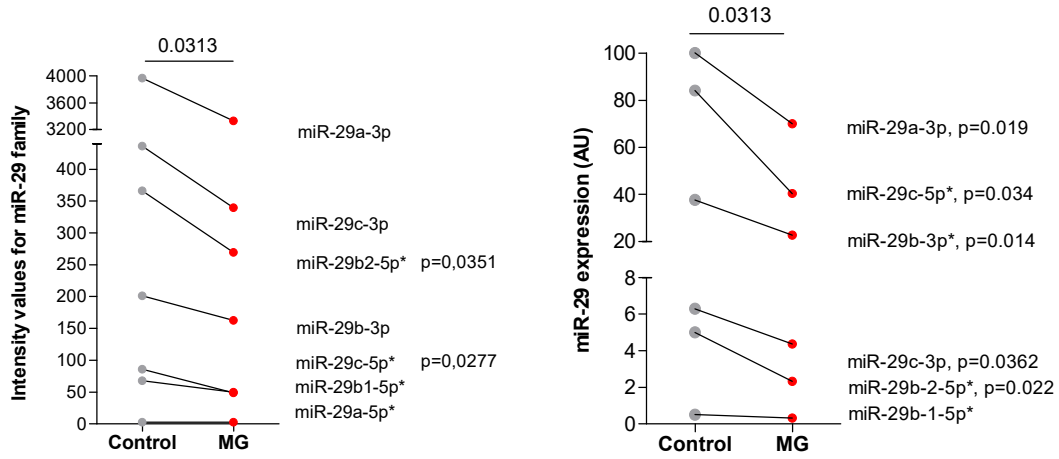
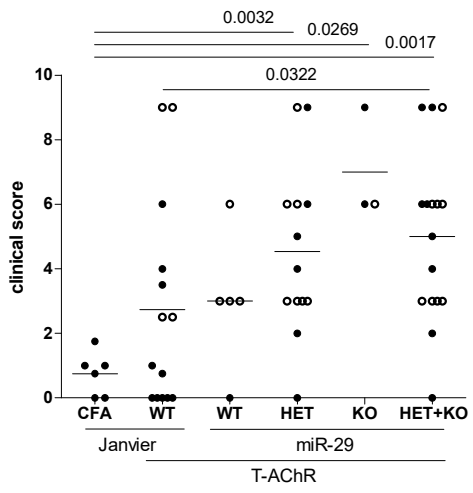


Figure 2

A



B

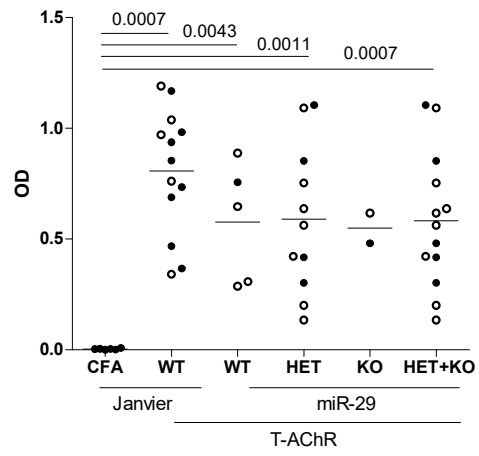


Table 1: Characteristics of patients whose thymus were used for RT-PCR experiments

(1) Degree of thymic hyperplasia: low hyperplasia (with 2 or fewer GCs per section) or high hyperplasia (with 3 or more GCs per section). (2) Clinical classification according to the Myasthenia Gravis Foundation of America (MGFA).

Patient ID	Gender	Age (years)	Degree of thymic hyperplasia (1)	Interval onset - thymectomy (months)	MGFA score at thymectomy (2)	Corticoid treatment	Cholinesterase inhibitors	Anti-AChR titer (nmol/L)
MG1	F	15	Low	13	III b	No	Yes	3.45
MG2	F	23	Low	7	II a	No	Yes	2118.7
MG3	F	29	Low	7	I a	No	NS	83.7
MG4	F	35	Low	24	IV a	No	Yes	11.1
MG5	F	20	Low	6	II a	No	Yes	>100
MG6	F	32	Low	6	II b	No	Yes	17.3
MG7	F	30	High	2	IV a	No	NS	>100
MG8	F	30	High	14	I	No	NS	3180.2
MG9	F	25	High	3	II a	No	Yes	3.21
MG10	F	28	High	4	II a	No	Yes	60.38
MG11	F	28	High	36	III a	No	Yes	9.7
MG12	F	22	High	2	III a	No	Yes	264

Table 2: List of TaqMan microRNA Assays used for Custom RT Pool and qPCR on miRNAs

Manufacturer's "Assay ID"	miRNA name	miRBase ID	Sequence
002112	hsa-miR-29a-3p	MIMAT0000086	UAGCACCAUCUGAAAUCGGUUA
002447	hsa-miR-29a-5p	MIMAT0004503	ACUGAUUUCUUUUGGUGUUCAG
000413	hsa-miR-29b-3p	MIMAT0000100	UAGCACCAUUUGAAAUCAGUGUU
002165	hsa-miR-29b-1-5p	MIMAT0004514	GCUGGUUUCAUAUGGUGGUUUAGA
002166	hsa-miR-29b-2-5p	MIMAT0004515	CUGGUUUCACAUGGUGGCUUAG
000587	hsa-miR-29c-3p	MIMAT0000681	UAGCACCAUUUGAAAUCGGUUA
001818	hsa-miR-29c-5p	MIMAT0004673	UGACCGAUUUCUCCUGGUGUUC

ARTICLE 3

Causes and consequences of miR-150 dysregulation in Myasthenia Gravis

Article submitted

Objectives

miRNAs are often described as biomarkers in many diseases, in particular in cancer and in autoimmunity. Indeed, in these diseases, disease progression and monitoring are necessary to ensure a good care of the patients. To this purpose, miRNAs are good candidates as they are very stable and easily recoverable in body fluids. miR-150-5p was previously characterized as a biomarker in MG due to its up-regulation in MG serum and its correlation with the improvement of symptoms after thymectomy. In this study, we investigated the causes and consequences of the miR-150 serum increase in the thymus of MG patients but also in peripheral cells. In the thymus, we showed that miR-150 is overexpressed and especially in highly hyperplastic thymuses because of the abnormal presence of B cells, organized in germinal centers. We also showed that among PBMCs, miR-150 is down-regulated and especially by CD4⁺ T cells. Therefore, serum miR-150 could be released from hyperplastic thymuses or secreted from activated CD4⁺ T cells. We also investigated the effects of miR-150 on peripheral cells by culturing them with miR-150 and anti-miR-150 oligonucleotides. We demonstrated that miR-150 is able to modulate its target genes, including MYB, one of its major target involved in many processes in the immune system. After the anti-miR-150 treatment, we observed that CD4⁺ and CD8⁺ T cells were particularly subjected to death. Anti-miR-150 unlocked the negative regulation of pro-apoptotic genes controlled by miR-150. These results show that miR-150 effects can be reversed and that modulation of miRNAs must be done carefully.

In addition, these data show that miRNAs, through the example of miR-150, can have functional roles and be more than biomarkers.

Short title: Altered expression of miR-150 in Myasthenia Gravis

Causes and consequences of miR-150-5p dysregulation in Myasthenia Gravis

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Abbreviations:	AChR:	acetylcholine receptor
	AIFM2:	apoptosis inducing factor, mitochondria associated 2
	GC:	Germinal center
	MG:	Myasthenia Gravis
	miRNA:	microRNA
	miR-150-5p:	miR-150
	P53:	tumor protein 53
	PBL:	peripheral blood lymphocyte
	PBMC:	peripheral blood mononuclear cell

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ABSTRACT

Objectives: miR-150-5p is characterized as a biomarker in Myasthenia Gravis (MG) with anti-acetylcholine receptor antibodies. miR-150 expression is increased in the serum of patients and decreased after thymectomy, correlated with an improvement of symptoms. We investigated the implication of the thymus and peripheral cells in increased serum levels of miR-150 in MG and its consequences.

Methods: The expression of miR-150 and *MYB* was analyzed by RT-PCR in thymic samples and peripheral cells from early-onset MG patients and non-MG controls. Thymic expression of miR-150 was further studied by *in situ* hybridization. Next, the *in vitro* effects of miR-150 or anti-miR-150 were investigated on peripheral cells. The expression of target genes of interest was analyzed by RT-PCR, western blot and flow cytometry.

Results: In MG thymuses, miR-150 was overexpressed in correlation with the abnormal presence of B cells, in particular B cells of the mantle zone of ectopic germinal centers. In contrast, in peripheral cells, miR-150 was down-regulated especially in CD4⁺ T cells. Next, we demonstrated that miR-150 decreased and anti-miR-150 increased *MYB* expression in peripheral lymphocytes. However, only anti-miR-150 caused a specific cell death of CD4⁺ and CD8⁺ T cells, by inducing the expression of pro-apoptotic genes normally controlled by miR-150.

Interpretation: These results showed that the increased serum levels of miR-150 could result from a release from MG thymuses but also from activated peripheral CD4⁺ T cells. In addition, we demonstrated that altered miR-150 levels clearly modulated the expression of target genes and peripheral cell survival.

INTRODUCTION

Myasthenia Gravis (MG) is an autoimmune disease due to antibodies against several components of the neuromuscular junction. Patients suffer from more or less invalidating muscle weaknesses leading to a generalized fatigability ¹. The majority of patients (85%) display antibodies against the acetylcholine receptor (AChR), and in the early-onset form of the disease, functional and morphological thymic abnormalities are frequently observed. They are mainly characterized by active neoangiogenic processes ^{2, 3} leading to B-cell infiltrations associated with the ectopic development of germinal centers (GCs): thymic hyperplasia ⁴. There is no global correlation between the AChR antibody titer and the severity of the disease, although such a correlation has been described at the individual level ⁵. A clear correlation exists between the anti-AChR antibodies serum level and the degree of thymic hyperplasia⁶. Moreover, the number of GCs is reduced in patients undergoing corticosteroid treatment ⁷. All these observations support the role of the thymus in the pathogenesis of MG, and thymectomy is often advised for AChR-MG patients to improve symptoms ⁸.

In collaboration with Dr Punga's group, we investigated microRNAs (miRNAs) as potential circulating biomarkers in early-onset AChR-MG patients. We observed that miR-150-5p (miR-150) is upregulated in the serum of AChR-MG patients and its expression is lowered after thymectomy, accompanied by an improvement of symptoms ⁹. Another study on late-onset MG patients, showed an decreased expression of circulating miR-150 in correlation with a better MG patients' clinical status ¹⁰.

First described in 2007 by Zhou *et al.* in hematopoietic stem cells ¹¹, miR-150 is described as an immuno-miR regulating immune functions, such as proliferation, apoptosis and differentiation of NK, T and B cells ¹¹⁻¹⁵. miR-150 is also a marker of lymphocyte activation ¹⁶ and is highly sensitive to stress signals such as lipopolysaccharide or glucocorticoids ^{17, 18}. As

in early-onset MG patients, miR-150 serum level is increased in various immune pathologies as in multiple sclerosis¹⁹, during HIV infection²⁰ but also in some cancers^{21, 22}. Of interest, miR-150 is also overexpressed in inflammatory organs, as for example in salivary glands in Sjögren Syndrome²³ and in kidneys in lupus nephritis²⁴. Overall, miR-150 is thought to be a sensor of general lymphocyte activation induced by inflammation²⁵.

In this study, we investigated the expression levels of miR-150 in the thymus and in peripheral blood cells of MG patients in order to better understand the increase observed in the serum. We observed that miR-150 was increased in the MG thymus in correlation with the presence of B cells, and decreased in peripheral blood cells, especially in CD4⁺ T cells. In addition, we demonstrated that serum level of miR-150 could have a functional role on peripheral blood cells by controlling the expression of MYB or pro-apoptotic genes, such as P53 and AIFM2.

METHODS

Thymic and blood samples

Thymic biopsies were obtained from the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France), where early-onset MG patients (12-44 years old, n=43 with details given in Table 1A) underwent thymectomy and age/sex-matched non-MG controls (15-33 years old, n=21) cardiovascular surgery. MG thymuses were classified according to the degree of follicular hyperplasia assessed by Marie Lannelongue Surgical Center pathologists (high degree of hyperplasia with 3 or more GCs per section vs. low degree of hyperplasia with 2 or less GCs per section). Thymic epithelial cells were extracted from control and MG thymic biopsies and were used for real-time PCR (RT-PCR) as previously described ²⁶.

Blood was collected from MG patients (19-65 years old, n=26, Table 1B) at the time of the thymectomy and from control donors by the French Blood Establishment (Établissement Français du Sang, EFS) (22-65 years old, n=28). MG patients did not display any thymoma. All patient information are displayed on table 1A-B and studies on blood and thymic samples were approved by local ethics committees (RCB 2006-A00164-47 and RCB 2010-A00250-39).

***In situ* hybridization**

In situ hybridization was performed on formalin-fixed paraffin-embedded (FFPE) thymic tissues (3 adults controls and 3 MG donors (Table 1A)) using a semi-automated method previously described²⁷. Briefly, 5µm-thick FFPE thymic tissues on polarized glass slides (Menzel-Gläzer SuperFrost Plus, Thermo Scientific, Villebon-sur-Yvette, France) were placed in xylene and hydrated using ethanol-descending concentration baths. Tissue permeabilization was done using protease-K before dehydration of slides by placing them in ethanol-increasing concentration baths. Previously diluted and denatured double-DIG-LNA

probes (Exiqon, Vedbaek, Denmark) for miR-150-5p (#38053-15) and for the scramble miRNA (#99004-15) were placed directly on slides and incubated in a hybridizer (Dako-Agilent, Santa Clara, USA) for 2h at miRNA hybridization temperature. After several stringent washes, the automated protocol was applied. This protocol includes an incubation with peroxidase inhibitor and a blocking step with casein followed by an incubation with mouse anti-DIG primary antibody. Signal detection was performed by OptiviewDAB Detection Kit (#760-700, Ventana Medical Systems, Tucson, USA) and OptiView Amplification Kit (#760-099). Finally, slides were dehydrated and mounted to visualize hybridization with a bright-field microscope.

Peripheral blood mononuclear cells (PBMCs) and cell sorting

PBMCs from MG patients (Table 1B) and healthy controls were isolated from fresh whole blood, collected in EDTA tubes, using the Ficoll technique ²⁸. Cells were stored in fetal calf serum containing 20% DMSO and kept in liquid nitrogen until use. To assess miR-150 expression in different circulating cell sub-populations, CD19⁺ B and CD4⁺ T cells were successively sorted with EasySep kits (#18054, #18052, Stem Cell, Vancouver, Canada) from some freshly collected PBMCs of MG and healthy donors. Cells were next used for RNA extraction. Altogether 26 MG donors were use (Table 1B) for PBMCs and/or CD19⁺ B cells and CD4⁺ T cells).

For PBMC cultures, cells from healthy donors were seeded in X-vivo medium (Lonza, Levallois-Perret, France) in 96-well plates (1.10⁶ cells per well). Next cells were treated with a non-toxic dose of 10μM of miR-150 or a scramble miRNA (Eurogentec, Seraing, Belgium), or next 10μM antimiR-150. The natural uptake of naked oligonucleotides by the cells is named gymnosis and occurs without creating pores by transfection or other chemical reagents and physical techniques ²⁹. antimiR-150 is an unmodified miRNA specifically targeting miR-150 in order to

inhibit it. miR-150, anti-miR-150 and scramble miRNA sequences are available in Table 2A. After 72h, non-adherent peripheral blood lymphocytes (PBLs) were collected and used for quantitative PCR (qPCR), Western blot or flow cytometry analyses.

RNA extraction

Total RNA was extracted from thymic biopsies or cells with the mirVana miRNA Isolation Kit in particular for RT-PCR on miRNAs or by TRIzol for mRNA analyses (Life Technologies, Villebon-sur-Yvette, France). Biopsies were first lysed in the Lysis/Binding buffer provided in the mirVana kit with the FastPrep FP120 instrument (Qbiogen, Illkirch, France) whereas cells were directly lysed in TRIzol after culture. RNA quality was assessed on a Bioanalyzer 2100 (Agilent Technologies, Les Ulis, France).

RT-PCR for miRNAs

miRNAs were retro-transcribed from total RNA using the Universal cDNA Synthesis Kit II (Exiqon), according to the manufacturer's instructions. cDNAs were next diluted to 1:20 in nuclease-free water prior to qPCR reactions that were carried out using the ExiLent SYBR Green master mix (Exiqon) on a LightCycler 480 (Roche, Meylan, France). PCR settings were as followed: 1 cycle of polymerase activation and denaturation at 95°C for 10 minutes, 45 cycles of amplification at 95°C for 10 seconds and 60°C for 1 minute, with a ramp-rate of cooling of 1,6°C/s. miRNA expression was normalized to 28S rRNA expression. miRNA sequences used for RT-PCR are listed on Table 2A.

RT-PCR for mRNAs

mRNAs were retro-transcribed from 1µg of total RNA using the Reverse Transcriptase AMV kit (Roche) and qPCR experiments were carried out using LightCycler 480 SYBR Green Master Mix

(Roche) according to the manufacturer's instructions. qPCR cycle conditions were: 1 cycle of polymerase activation and denaturation at 95°C for 10 minutes, 45 cycles of amplification at 95°C for 10 seconds, 60-64°C for 1 minute and 72°C for 14 seconds. Primer sequences are listed on Table 2B.

Flow cytometry

PBMCs were surface-stained with anti-human monoclonal antibodies for 45 minutes at 4°C: anti-CD4-APC (clone RPA-T4; #17-0049-42, eBioscience, Paris, France), anti-CD8-PeCy7 (clone RPA-T8; #557750, BD Biosciences, Le-Pont-de-Claix, France) and anti-CD19-ef450 (clone HIB19; #48-0199-42, eBioscience) antibodies. Cells were fixed and permeabilized with the Intracellular Fixation and Permeabilization buffer set (#00-5523-00, eBioscience) according to the manufacturer's instructions. Intracellular MYB was stained with an anti-MYB polyclonal antibody (#AF6209, R&D Systems). Live/dead staining (Fixable Near-IR Dead Cell Stain Kit, #L10119, Life Technologies) was done to gate on viable cells and to analyze cell death. A Ki-67-PeCy7 antibody (clone SolA15; #25-5698-82, eBioscience) was used to assess cell proliferation. Flow cytometry was performed on a FACS Canto II (BD Biosciences) and data were analyzed using FlowJo software.

Western Blot

Whole-cell proteins from PBLs were extracted in RIPA buffer (20mM Tris HCl pH 8, 137mM NaCl, 10% Glycerol, 1% NP40, 2mM EDTA) and cellular debris were eliminated after centrifugation at 12 000 g for 25 minutes at 4°C. Protein concentration was assessed using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. 15µg of proteins were diluted in Laemmli buffer, separated on polyacrylamide gels (NuPAGE 4-12% Bis-Tris Gel, Life Technologies) and transferred to a PVDF membrane

(Immobilon-P, Merck-Millipore, Darmstadt, Germany). Transfer efficiency was evaluated with Ponceau staining. Next, the membrane was saturated using 5% non-fat milk diluted in TBS 0.05% Tween (TBS-T) for 1 hour, incubated with mouse anti-human MYB monoclonal antibody (2 µg/mL, #05-175, Merck-Millipore) overnight at 4°C. After several washes in TBS-T, the membrane was incubated with HRP sheep anti-mouse secondary antibody (#NA931, GE Healthcare, United Kingdom) for 1 hour at room temperature. Finally, the membrane was washed in TBS-T and revealed with electrochemiluminescence (ECL) on autoradiography films (Amersham, GE Healthcare). MYB intensity was normalized on β-actin (#612656, BD Transduction Laboratories) expression. Band intensity quantification was done using ImageJ software.

Statistical analysis

In correlation analysis, a Pearson's correlation test was applied and significance was assessed. In dot plot graphs, we used the non-parametric Mann Whitney test or the Wilcoxon paired test to do 2-by-2 comparisons, as indicated in figure legends.

RESULTS

Increased miR-150 expression in highly hyperplastic thymus

miR-150 expression levels were assessed in the thymus of early-onset MG patients displaying different degrees of follicular hyperplasia characterized by the number of GCs. miR-150 was particularly increased in patients with a high degree of thymic hyperplasia compared to controls and to patients with a low degree of hyperplasia. However, in corticoid-treated patients, with a low or no hyperplasia, the levels of miR-150 were not reduced compared to MG patients with a high degree of hyperplasia (Figure 1A). Even if corticoids are well known to decrease the number of GCs⁷, corticoids have also the ability to induce miR-150 expression as described by Palagani *et al.*¹⁸.

As we observed a significant overexpression of miR-150 in patients with a high degree of thymic hyperplasia, we analyzed the expression of *CD19* mRNA, a B-cell marker, in correlation with miR-150 expression for untreated-MG patients. We demonstrated a significant correlation between *CD19* mRNA and miR-150 expression (Figure 1B). As they represent one of the major cell population in the thymus, we also assessed miR-150 expression in thymic epithelial cells but did not see any differences between controls and MG patients (Figure 1C). In parallel, we analyzed in untreated MG patients the expression levels of *MYB* mRNA, one of miR-150 best-known and major target. We observed that *MYB* was significantly down-regulated in the thymus of MG patients with a high degree of hyperplasia. More, we showed that *MYB* expression was inversely and significantly correlated with miR-150 expression in the thymus of MG patients (Figure 1E).

Altogether, these results suggest that miR-150 overexpression in MG thymuses is due to B cells and to the presence of ectopic GCs, and that increased expression of miR-150 could modulate intrathymic expression of mRNA targets such as *MYB*.

Strong miR-150 expression in B cells of the mantle zone of germinal centers

In order to visualize more precisely the localization of miR-150 in the thymus, we performed *in situ* hybridization in control (Figures 2A-B) and untreated-MG (Figures 2C-F) thymuses. A miR-150 labeling was observed associated with thymocytes in both the cortical and medullary regions, and miR-150 distribution seemed similar in adult control and MG patients (Figures 2A-D).

However, in the thymus of MG patients, we observed a strong labeling for miR-150 preferentially located in the mantle zone around GCs (Figures 2E-F). These data demonstrated that the higher expression of miR-150 expression as observed by PCR in the hyperplastic thymus of untreated MG patients was linked to B cells and ectopic GCs.

Decreased expression of miR-150-5p in peripheral CD4⁺ T cells

Circulating miR-150 is overexpressed in the serum of MG patients ⁹ and we further investigated miR-150 expression in PBMCs from MG patients. Interestingly, miR-150 was found significantly less expressed in MG PBMCs compared to control PBMCs (Figure 3A). This decrease could not be attributed to a loss of a peripheral cell subpopulation, as no major changes have never been observed in MG patients. Among PBMCs, we investigated the expression of miR-150 in isolated CD4⁺ T cells (Figure 3B) cells and CD19⁺ B (Figure 3C), the cells mainly involved in the autoimmune response in MG. miR-150 was found decreased in CD4⁺ T cells but not in CD19⁺ B cells (Figures 3B-C). For a few donors, miR-150 expression was also assessed in CD14⁺ monocytes but no difference between MG and control was observed

(data not shown). These data suggest that the increase of miR-150 in the serum of MG patients could be linked to a release from CD4⁺ T cells.

Analyzing *MYB* mRNA expression in PBMCs, we were surprised to observe a significant down-regulation of its expression in PBMCs from MG patients. We hypothesize that miR-150 could be preferentially secreted by activated T cells without affecting directly endogenous mRNA. This could explain why the intracellular decrease of miR-150 was not directly associated with an increased expression of its target, as shown by de Candia *et al.*¹⁶.

miR-150 decreased *MYB* expression in peripheral blood cells

To investigate if circulating miR-150 could have a function in MG and other inflammatory diseases, we analyzed the impact of miR-150 on peripheral circulating cells by treating control PBMCs with miR-150, as detailed in the method section. To work in physiological conditions, cells were not transfected, and miR-150 was added in cell culture medium and “naturally” delivered into cells thanks to the gymnosis process. Non-adherent PBLs were recovered after 72h. We validated by qPCR that miR-150 level was elevated intracellularly in cells treated with miR-150 compared to cells treated with the scramble miRNA (Figure 4A). Then, we demonstrated at the mRNA (Figure 4B) and protein (Figures 4C-D) levels that MYB was less expressed in cells treated with miR-150. The decreased expression of MYB upon miR-150 treatment was also confirmed by flow cytometry after the exclusion of dying cells and by gating on lymphocytes, as detailed in figure legend (Figures 4E-F). Next, we analyzed by flow cytometry the effect of anti-miR-150, at the same concentration than miR-150 and the scramble miRNA, on MYB expression. We confirmed by qPCR that miR-150 expression was decreased in PBLs upon anti-miR-150 treatment (Figure 4G). By flow cytometry, we showed that anti-miR-150 had an opposite effect compared to miR-150 and significantly increased MYB

expression in lymphocytes (Figures 4E-H). Altogether, these results demonstrate that increased level of miR-150 in the serum could penetrate circulating cells and decrease the expression of miR-150 target genes, such as *MYB*. An anti-miR-150 treatment by decreasing the levels of endogenous miR-150 could have the opposite effect on target gene expression.

Increased cell death associated with miR-150 inhibition

We also investigated the global effect of miR-150 or anti-miR-150 on lymphocytes after 72h treatments. The addition of miR-150 tends to decrease the proportion of lymphocytes but it was not statistically significant (Figure 5A) and we did not observe any effects of miR-150 on cell proliferation or cell death (Figures 5B-C). In contrast, anti-miR-150 significantly decreased the percentage of lymphocytes (Figure 5A). This was not associated with a decreased proliferation of cells but with an increase of cell death (Figures 5 B-C). We next analyzed if CD4⁺ T cells, CD8⁺ T cells and CD19⁺ B cells were similarly impacted by anti-miR-150. We observed that anti-miR-150 increased with a two-fold factor CD4⁺ and CD8⁺ T-cell death while it did not affect CD19⁺ B cells (Figures 5G-I).

These results suggested that miR-150 was playing a role in the survival of CD4⁺ and CD8⁺ T cells and the inhibition of miR-150 could induce the expression of pro-apoptotic genes. We identified four pro-apoptotic genes targeted by miR-150 in different prediction databases (miRTarBase, miRWalk, Diana-microT and TargetScan): *P53* (Tumor Protein 53), *AIFM2* (Apoptosis Inducing Factor Mitochondria associated 2), *SP1* (Specificity Protein 1) and *PDCD4* (Programmed Cell Death 4). We analyzed the effect of anti-miR-150 and miR-150 on the expression of these genes. *PDCD4* expression was not modified by any treatments and *SP1* PCR was not effective. However, *P53* and *AIFM2* mRNA expression levels were increased upon the anti-miR-150 treatment compared to the scramble miRNA treatment. We did not see any

down-regulation of *P53* and *AIFM2* expression upon miR-150 treatment (Figure 5J). This suggests that the basal level of miR-150 in control conditions was sufficient to repress the expression of *P53* and *AIFM2*, and adding more miR-150 did not increase this repression.

As upon miR-150 treatment, we observed a decreased expression of *MYB* (Figure 4) and a slight decrease in the percentage of lymphocytes (Figure 5A), we analyzed the effect of miR-150 on the expression of two anti-apoptotic genes, *BCL-2* (B-cell leukemia/lymphoma 2) and *BCL-XL* (also called BCL2L1: BCL-2 like 2) that are induced by *MYB*³⁰. Nevertheless, we did not observe any decreased expression of *BCL-2* and *BCL-XL* mRNAs in PBMCs treated with miR-150 (data not shown). Altogether, these results underline that miR-150 and anti-miR-150 did not always have opposite effects and can modulate specifically cell behavior.

DISCUSSION

We previously showed that miR-150 is increased in the serum of early-onset AChR-MG patients. Its expression is lowered after thymectomy and is correlated with an improvement of the symptoms. Here, we investigated the expression level of miR-150 in the thymus and peripheral blood cells of MG patients to understand why miR-150 is increased in the serum of MG patients and what are the consequences of miR-150 fluctuations on peripheral cells.

miR-150 was overexpressed in hyperplastic MG thymuses

Here, we show that miR-150 was more expressed in the thymus of MG patients compared to healthy controls, and in particular in patients displaying a high degree of thymic hyperplasia. Gualeni *et al.* demonstrated that in reactive lymph nodes, miR-150 is expressed by small lymphocytes of primary follicles and of the mantle zone around GCs²⁷. In addition, Tan *et al.* also observed miR-150 to a lesser extent in a few cells in the dark zone of GCs, a dense zone of proliferating B cells³¹. We observed a similar expression pattern in ectopic GCs in the thymus of MG patients with miR-150 mainly expressed by naïve B cells, resident cells of the mantle zone. These observations led us to conclude that the increased expression of miR-150 in hyperplastic MG thymuses was linked to the abnormal presence of B cells and in particular to the development of GCs. We observed that the increased expression of miR-150 in MG thymuses was associated with a decreased expression of *MYB*, the most well-known miR-150 mRNA target that displays four binding sites. *MYB* is a transcription factor essential for hematopoiesis and is highly expressed in the thymus³². *MYB* is also characterized as an early regulator of T-cell associated diseases with an altered expression in autoimmune diseases³³. In the MG thymus, miR-150 could be secreted by B cells and alter *MYB* expression locally and consequently affect T cells, knowing that the T-cell repertoire is altered in MG³⁴. Indeed, *MYB*

is known to be involved at several stages in thymopoiesis: transition through the double-negative 3 stage, survival of CD4⁺CD8⁺ thymocytes, and differentiation of CD4⁺ thymocytes³⁵. As miR-150 is highly expressed in hyperplastic thymuses, the question is whether the serum increase of miR-150 in MG is linked to a release from the thymus that would explain the serum decrease after thymectomy⁹. To support this hypothesis, we should observe a higher serum level of miR-150 in MG patients with highly hyperplastic thymus. A recent study analyzing the serum levels of miR-150 in MG patients together with the degree of thymic hyperplasia did not observe any correlation³⁶. However, in this study, 79% of patients were under corticoid therapy. Corticoids strongly induce miR-150 expression in the thymus or in corticoid-sensitive cell lines^{17,18} and even if they are known to decrease the number of thymic GCs, we observed an increased expression of miR-150 in the thymus of cortico-treated MG patients, even with a low or no thymic hyperplasia. However, the link between the serum and thymic levels of miR-150 is also suggested by observations from other autoimmune diseases. Indeed, increased expression of miR-150 is often observed both in the serum and inflammatory organ characterized by ectopic lymphoid infiltrations, such as in Sjögren Syndrome^{23,37} and in lupus nephritis^{24,38}.

miR-150 is down-regulated in CD4⁺ T cells in MG patients

In this study, we also showed that miR-150 was less expressed in PBMCs from MG patients compared to controls, demonstrating that circulating and cellular miR-150 levels could vary in opposite ways. This was also observed in other pathological conditions. In HIV/AIDS patients, miR-150 expression is increased in the serum and decreased in PBMCs in symptomatic patients. While the decrease in PBMCs is clearly correlated with the loss of CD4⁺ T cells, the increase in the serum is not well understood²⁰. In chronic lymphocytic leukemia,

Stamatopoulos *et al.* also observed an increase in serum level of miR-150 and a cellular decrease during disease progression. They suggest that cellular miR-150 could be regulated by its release into the extracellular space³⁹. In addition, similarly to what we observed in MG, different studies demonstrated that miR-150 is decreased in PBMCs from Sjögren Syndrome patients while it is increased in the serum of patients and in inflammatory salivary glands^{23, 37, 40}.

Analyzing miR-150 expression in cell subtypes, we observed that the decrease in PBMCs from MG patients was mainly due to CD4⁺ T cells. Similarly, in autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, miR-150 is less expressed in CD3⁺ T cells, and in particular CD4⁺ T cells^{41, 42}. In healthy donors, miR-150 was reported to be less expressed in activated CD4⁺ T cells compared to resting cells^{43, 44}. De Candia *et al.* demonstrated that upon *in vitro* activation lymphocytes down-regulate intracellular miR-150 that accumulate in exosomes¹⁶. In addition, they also showed that miR-150 expression level remained practically unchanged in the serum of CD4⁺ T cell-depleted mice after stimulation²⁵, suggesting that miR-150 expression is mediated through CD4⁺ T cells. Consequently, we hypothesize that increased serum level in MG patients could be due to the release of miR-150 by activated CD4⁺ T cells in MG.

By analyzing *MYB* expression in PBMCs, we observed that while miR-150 was decreased in PBMCs from MG patients, its main target *MYB* was also surprisingly down-regulated. This observation support again the fact that miR-150 is probably released directly upon cell activation. Indeed, miR-150 belongs to a category of miRNAs that are mainly exported and enriched in extracellular vesicles⁴⁵ even if it can also be detected circulating associated with non-vesicular AGO2 ribonucleoprotein complexes⁴⁶. We hypothesized that in MG patients, miR-150 could act as an “endocrine” miRNA on neighboring cells⁴⁷. Upon production, miR-

150 would be directly released extracellularly without affecting endogenous mRNA expression and the serum increase of miR-150 could therefore down-regulate *MYB* expression in surrounding PBMCs. Of interest, *MYB* is known to be required for regulatory T cells homeostasis in periphery as *MYB* deletion in these cells resulted in the development of multi-organ autoimmune diseases ⁴⁸.

miR-150 modulated peripheral cell behavior

Most miRNAs described in diseases are characterized only as biomarkers and their functional roles are not further investigated. Here, we analyzed the effects of miR-150 when added on peripheral blood cells. By looking at its major target, *MYB*, we showed by different approaches that *in vitro* miR-150 was able to decrease *MYB* expression at the mRNA and protein levels. As reviewed by Greig *et al.*, *MYB* plays a critical and complex role in hematopoietic cells, promoting cell proliferation but also cell differentiation ⁴⁹. Nevertheless, if the treatment of PBMCs with miR-150 decreased *MYB* expression, in our *in vitro* experiments it did not affect the proliferation or the cellular death of CD4, CD8 and CD19 cells.

We also investigated the effect of an anti-miR-150, designed to neutralize miR-150 and to cancel its effects. As expected, we demonstrated that anti-miR-150 had an antagonistic effect on the expression of *MYB* compared to miR-150. Moreover, we also observed that anti-miR-150 induced cellular death of CD4⁺ and CD8⁺ T cells. We showed that two pro-apoptotic genes, *P53* and *AIFM2*, directly regulated by miR-150, were upregulated when cells were treated with anti-miR-150. These results suggest that CD4⁺ and CD8⁺ T-cell death could be due to the overexpression of pro-apoptotic genes tightly controlled in the first place by miR-150. Next step would be to investigate if specific T-cell subsets are more sensitive to apoptosis in response to anti-miR-150.

Interestingly, *P53* and *AIFM2* were not repressed upon miR-150 treatment. The sensitivity of target genes to the addition of miR-150 or anti-miR-150 could be linked to the number of miRNA target sites. Indeed, *MYB* mRNA displays 4 fixation sites for miR-150: its expression is decreased upon miR-150 treatment and increased upon anti-miR-150 treatment. However, *P53* and *AIFM2* mRNAs display only one fixation site. Consequently the basal level of miR-150 in control conditions was probably sufficient enough to completely repress the expression of *P53* and *AIFM2*, while the addition of anti-miR-150 unlocked the negative control of the expression of these two pro-apoptotic genes.

Conclusion

miR-150 level is increased in the serum of MG patients and down-regulated after thymectomy, along with an improvement of symptoms^{9,36}. In this study, we clearly demonstrated increased expression levels in the thymus of MG patients due to B cells and GC development. We also showed that miR-150 was strongly decreased in peripheral CD4⁺ T cells. We cannot precisely conclude if the increased serum level is associated with a release from hyperplastic MG thymuses or secreted by activated CD4⁺ T cells, or both. Nevertheless, we demonstrated that the basal level of miR-150 had a protective effect on CD4⁺ and CD8⁺ T cells by controlling the expression of pro-apoptotic genes. In addition, increased serum level of miR-150 in MG could play a central role by regulating *MYB* whose expression is an important regulator of T-cell related autoimmune diseases³³.

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The authors do not declare any conflict of interest.

Authorship

MAC and SM performed and analyzed the experiments, FT collected samples and provided patient information, AVG and AG conducted ISH experiments, EF and JG provided thymic biopsies, AB provided blood samples, SB-A, AVG, AG and SM read and revised the manuscript, MAC and RLP designed the study, analyzed the experiments and wrote the manuscript.

Figure legends

Figure 1: Increased expression of miR-150-5p associated with thymic hyperplasia in MG.

(A) miR-150 expression in the thymus of non-MG controls (n=6), MG patients with a low degree of thymic hyperplasia (Low-H, n=6) or with a high degree of thymic hyperplasia (High-H, n=6) and in corticoid-treated MG patients displaying low or no hyperplasia (Low/no-H; n=12). miR-150 expression was normalized on 28S expression. (B) Correlation analysis of miR-150 and *CD19* mRNA expression in the thymus of untreated MG patients (n=12) (C) miR-150 expression in medullary thymic epithelial cells from non-MG adults (n=12) and untreated MG patients (n=15). Thymic epithelial cells from High-H MG patients are represented with empty black dots. (D) *MYB* mRNA expression in controls (n=6) and untreated MG patients (Low-H, n=6; High-H, n=6). mRNA expression was normalized on 28S expression. (E) Inverse correlation analysis of miR-150 and *MYB* mRNA expression in the thymus of untreated MG patients (n=12). P-values were assessed by the Mann-Whitney test and only p-values <0.05 are indicated on the graphs. Pearson's correlation was assessed and determination coefficient and p-values are indicated on the graphs.

Figure 2. miR-150 was highly expressed in B cells of the mantle zone of GCs in MG thymuses.

Representative *in situ* hybridization of miR-150 in the cortex (A) and the medulla (B) of the thymus of a non-MG control and in the cortex (C), the medulla (D) and two different GC (E, F) of the thymus of MG patients. miR-150 was located in B cells of the mantle zone of ectopic follicle with expanded GC. 1-cm bar length represents 50 μ m for 20X magnification and 25 μ m for 40X magnification. All *in situ* hybridization experiments were done in formalin fixed paraffin embedded thymic tissue sections, as fully detailed in the method section.

Figure 3. Decreased expression of miR-150-5p in peripheral cells from MG patients.

miR-150 expression was assessed by qPCR in (A) total PBMCs (controls n=13, MG n=9), (B) CD4⁺ T cells (controls n=14, MG n=9) and (C) CD19⁺ B cells (controls n=11, MG n=12). miR-150 expression was normalized on 28S expression. (D) *MYB* expression was assessed by qPCR in total PBMCs (controls n=12, MG n=11) and normalized on 28S expression. p-values were assessed by the Mann-Whitney test.

Figure 4. miR-150-5p decreased *MYB* expression in peripheral blood cells *in vitro*.

(A) Representative experiment of miR-150 expression in PBLs treated with a scramble miRNA (-) or miR-150 (+). (B) PCR analysis of *MYB* mRNA expression in PBLs treated with a scramble miRNA (-) or miR-150 (+) in 3 different donors. *MYB* mRNA expression was normalized on *GAPDH* mRNA expression. (C, D) Western blot analyses of MYB protein expression in PBLs treated with a scramble miRNA (-) or miR-150 (+). (C) Western blot quantitative analyses of MYB protein in 5 different donors. MYB expression level was normalized on β -actin. (D) Two representative experiments of MYB protein expression as seen after Western blot revelation in two different donors. (E) Representative experiment of MYB expression in PBLs treated with miR-150 (orange), scramble miRNA (green) or anti-miR-150 (blue), assessed by flow cytometry. Control IgG is represented as a full grey histogram. Cells were first gated for singlet on FSC-H vs. FSC-A, and further analyzed according to their uptake of Live/Dead stain. Finally, lymphocytes were gated on SSC-A vs. FSC-A. (F) Geomean of MYB expression in the lymphocyte population (n=8), treated with a scramble miRNA (-) or miR-150 (+). (G) Representative experiment of miR-150 expression in PBLs treated with a scramble miRNA (-) or anti-miR-150 (+). (H) Geomean of MYB expression in the lymphocyte population (n=6),

treated with a scramble miRNA (-) or antagomiR-150 (+). (C, F, H) p-values were assessed by the Wilcoxon paired test and only p-values <0.1 are indicated.

Figure 5. Increased cell death due to miR-150 inhibition.

(A) Analysis of the effect of miR-150 and antimiR-150 on the percentage of lymphocytes. PBMCs from 9 control donors were cultured for 72h with a scramble miRNA (10 μ M), miR-150 (10 μ M) or antimiR-150 (10 μ M). PBLs were recovered and analyzed by flow cytometry. Cells were first gated for singlet on FSC-H vs. FSC-A and lymphocytes were gated on SSC-A vs. FSC-A. (B) Proliferation was measured in total lymphocytes after 72h of culture according to Ki67 labelling. (C) Cell death was determined in total lymphocytes after 72h of culture according to a live-dead staining. (D-F) To evaluate cell proliferation after 72h in culture, PBLs were labelled with anti-CD4, CD8, CD19 and Ki67 antibodies (n=5). (G-I) To evaluate cell death after 72h in culture, PBLs were labelled with anti-CD4, CD8, CD19 antibodies and a live-dead stain (n=6). (J) qPCR analyses of pro-apoptotic genes *P53*, *AIFM2* and *PDCD4* expression in PBLs. Cells from 2 control donors were cultured for 72h with a scramble miRNA, miR-150 or antimiR-150 (10 μ M). Gene expression levels were normalized on *GAPDH* expression. (A-I) p-values were assessed by the Wilcoxon paired test and only p-values <0.05 were indicated.

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Figure 1

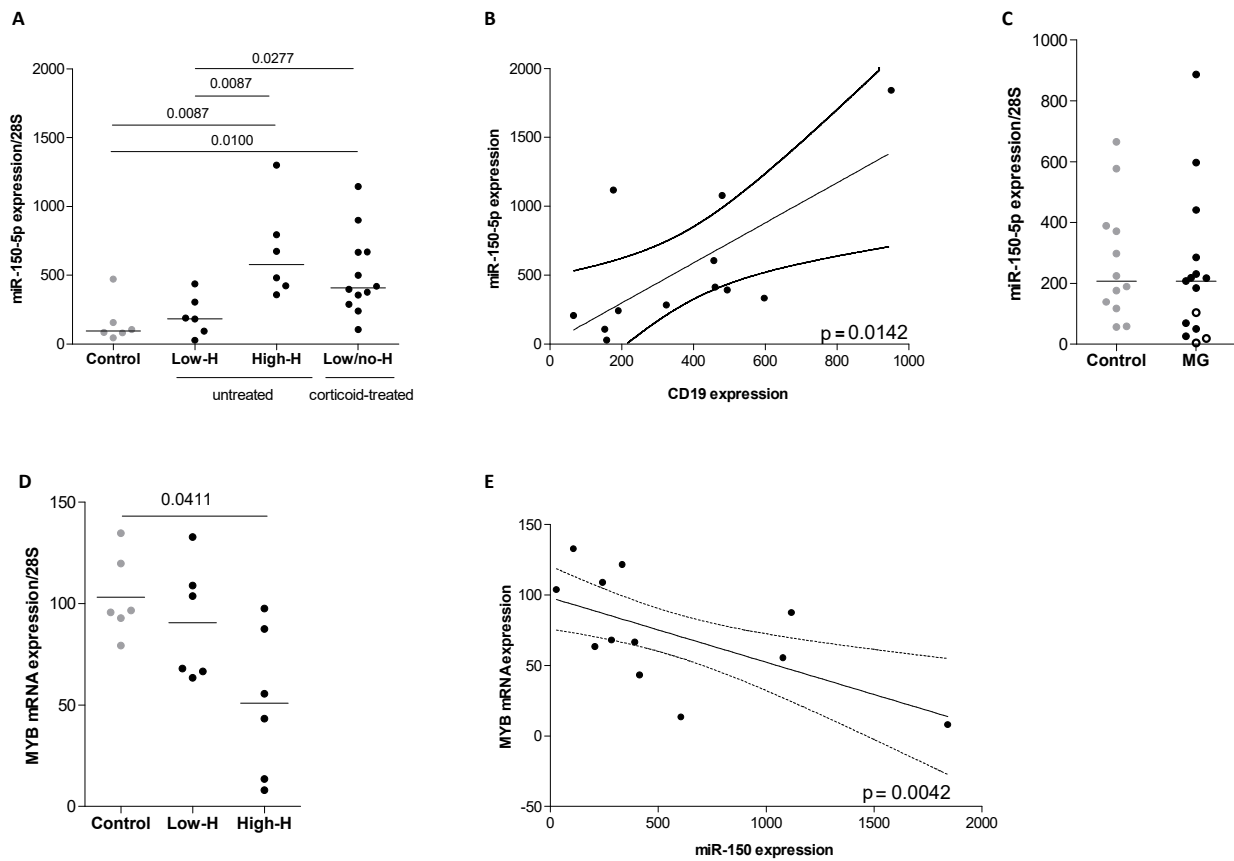


Figure 2

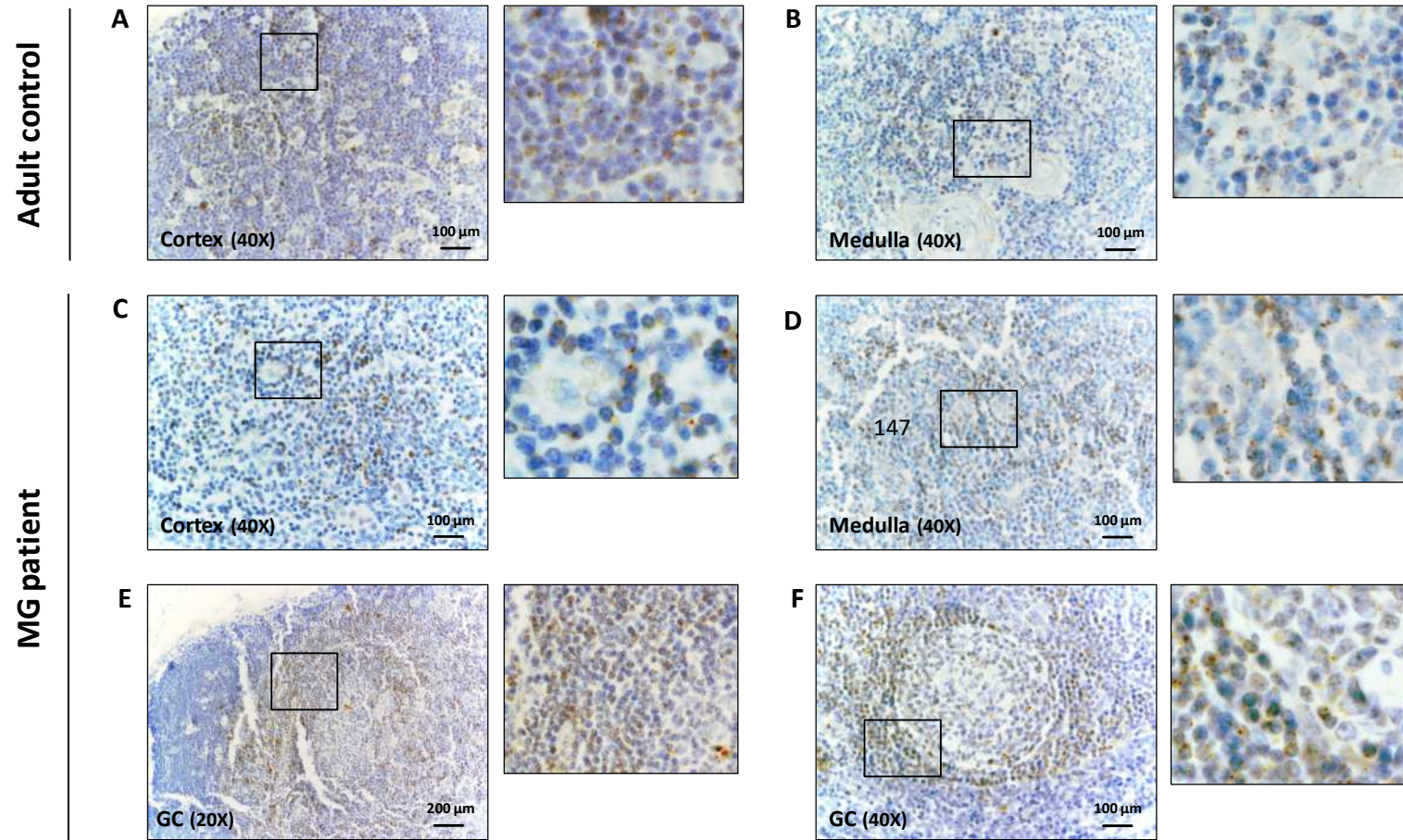


Figure 3

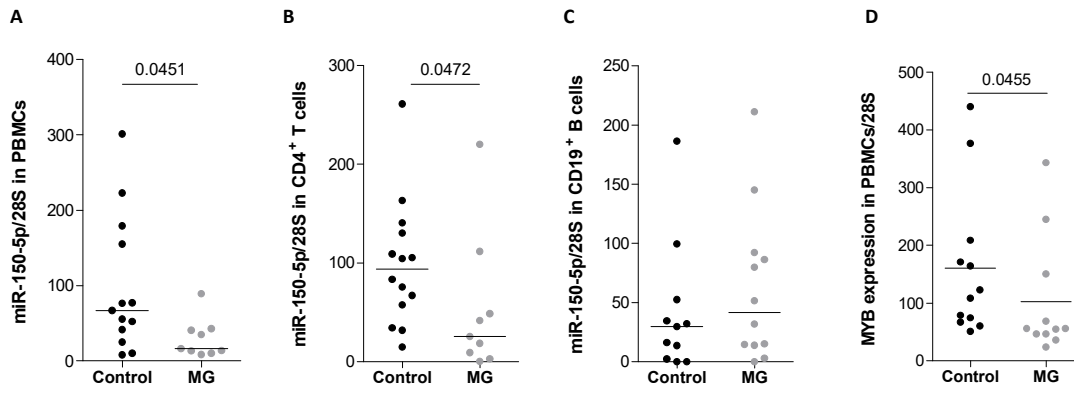


Figure 4

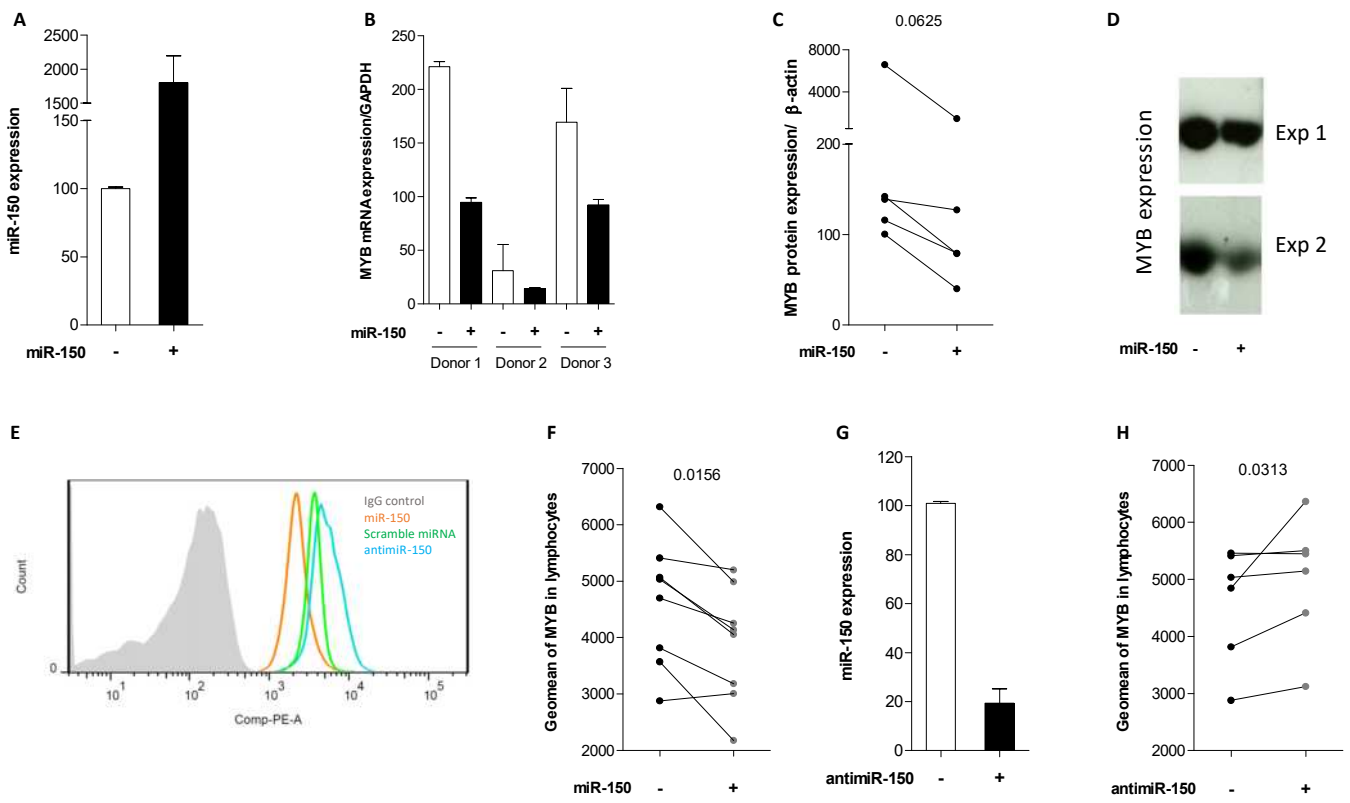


Figure 5

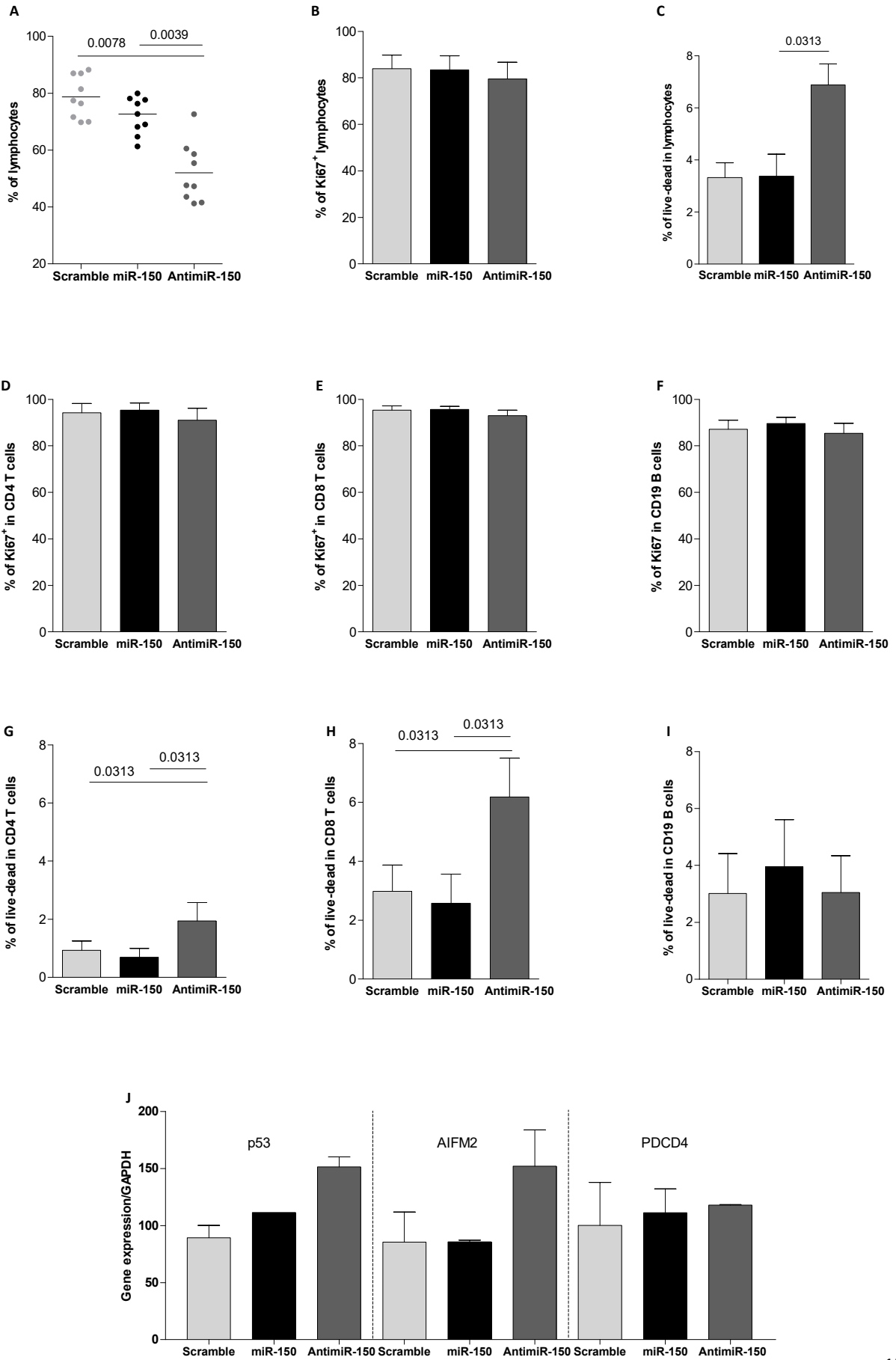


Table 1A: Characteristics of patients whose thymus and TECs were used for experiments

(1) Degree of thymic hyperplasia: low hyperplasia (with 2 or fewer GCs per section) or high hyperplasia (with 3 or more GCs per section). (2) Clinical classification according to the Myasthenia Gravis Foundation of America (MGFA). (3) Patients with corticoid treatment were only used for RT-PCR in Figure 1A. NS: not specified.

Patient ID	Gender	Age (years)	Degree of thymic hyperplasia (1)	Interval onset - thymectomy (months)	MGFA score at thymectomy (2)	Corticoid treatment (3)	Cholinesterase inhibitors	Anti-AChR titer (nmol/L)	PCR on thymic extracts	PCR on TECs	<i>In situ</i> hybridization
MG1	F	15	Low	13	III b	No	Yes	3.45	X		
MG2	F	23	Low	7	II a	No	Yes	2118.7	X	X	
MG3	F	29	Low	7	I a	No	NS	83.7	X		
MG4	F	35	Low	24	IV a	No	Yes	11.1	X		
MG5	F	20	Low	6	II a	No	Yes	>100	X		
MG6	F	32	Low	6	II b	No	Yes	17.3	X		
MG7	F	30	High	2	IV a	No	NS	>100	X	X	
MG8	F	30	High	14	I	No	NS	3180.2	X		
MG9	F	25	High	3	II a	No	Yes	3.21	X		
MG10	F	28	High	4	II a	No	Yes	60.38	X		
MG11	F	28	High	36	III a	No	Yes	9.7	X		
MG12	F	22	High	2	III a	No	Yes	264	X		
MG13	F	29	No hyperplasia	6	IV b	Yes	Yes	30.02	X		
MG14	F	19	No hyperplasia	18	III a	Yes	Yes	0.5	X		
MG15	F	33	No hyperplasia	30	II b	Yes	Yes	4.33	X		
MG16	F	37	No hyperplasia	6	III a	Yes	Yes	3.46	X		
MG17	F	23	Low	18	II b	Yes	Yes	NS	X		
MG18	F	35	No hyperplasia	18	V	Yes	Yes	6.9	X		
MG19	F	26	Low	24	II b	Yes	Yes	4.4	X		
MG20	F	32	NS	9	III a	Yes	Yes	9.6	X		
MG21	F	38	No hyperplasia	60	II b	Yes	Yes	21.4	X		
MG22	F	30	Low	10	II a	Yes	Yes	26.1	X		
MG23	F	44	No hyperplasia	12	II b	Yes	Yes	6.6	X		
MG24	F	31	No hyperplasia	30	II a	Yes	Yes	NS	X		
MG25	M	35	Low	3	III a	No	Yes	14		X	
MG26	F	12	High	18	IV a	No	Yes	>100		X	
MG27	F	18	Low	34	IV b	Yes	Yes	206.57		X	
MG28	F	23	Low	3	II a	No	Yes	>100		X	
MG29	F	34	High	90	NS	No	Yes	43		X	
MG30	F	38	Low	18	IV a	No	Yes	670		X	
MG31	F	42	Low	48	III b	No	Yes	0.38		X	
MG32	M	23	High	9	NS	No	Yes	NS		X	
MG34	F	38	Low	18	IV a	No	Yes	670		X	
MG35	F	29	Low	33	NS	Yes	Yes	>100		X	
MG36	M	20	Low	26	II	No	Yes	87.6		X	
MG37	M	24	Low	84	II b	No	Yes	35.3		X	
MG38	F	31	NS	24	NS	No	Yes	NS		X	
MG39	F	37	Low	NS	NS	No	Yes	NS		X	
MG40	F	17	Low	6	II a	No	Yes	1.1		X	
MG41	F	28	High	4	II a	No	Yes	60.38			X
MG42	F	30	High	2	IV a	NS	NS	>100			X
MG43	F	25	High	3	II a	No	Yes	3.21			X

Table 1B: Characteristics of patients whose PBMCs were used for experiments

Patient ID	Gender	Age (years)	Degree of thymic hyperplasia (1)	Interval onset - thymectomy (2)	MGFA score at thymectomy (3)	Corticoid treatment (4)	Cholinesterase inhibitors	Anti-AChR titer (nmol/L)	PCR on PBMCs	PCR on CD4 ⁺ T cells	PCR on CD19 ⁺ B cells
MG44	F	39	NS	NS	I	No	No	2.48	X		
MG45	F	43	NS	24	NS	No	No	>100	X		
MG46	F	21	Low	1	II b	No	Yes	1.82	X		
MG47	F	33	Low	96	II b	No	Yes	29	X		
MG48	F	32	Low	NS	I	No	No	>100	X		
MG49	F	65	Low	NS	II b	No	Yes	9	X		
MG50	F	44	Low	NS	I	No	Yes	1.1	X		
MG51	F	19	Low	1	II b	No	Yes	1.04	X		
MG52	F	57	NS	NS	NS	NS	NS	1.6	X		
MG53	F	44	NS	NS	III b	NS	NS	NS	X		
MG54	F	26	High	12	II b	No	Yes	8	X		
MG55	F	30	Low	54	II a	No	Yes	117.9	X		
MG56	F	23	High	16	III b	No	Yes	65.9	X		
MG57	F	20	Low	7	II a	No	Yes	>100	X	X	X
MG58	F	23	High	24	II b	No	Yes	10.9	X	X	X
MG59	F	19	Low	6	II a	No	Yes	12.4	X	X	X
MG60	M	33	Low	24	II b	No	Yes	7	X	X	X
MG61	F	29	Low	12	II b	No	Yes	48		X	X
MG62	F	29	Low	1	II a	No	Yes	1.6		X	X
MG63	F	41	Low	9	II b	No	Yes	1.67		X	X
MG64	F	21	Low	4	II b	No	Yes	1.82		X	X
MG65	F	28	High	18	II b	No	Yes	>100	X	X	
MG66	F	19	High	3	II b	No	Yes	23.3	X		X
MG67	M	24	Low	6	NS	Yes	Yes	1.46	X		X
MG68	F	28	Low	156	II b	No	Yes	2.3			X
MG69	F	22	High	3	II b	No	Yes	5.8			X

Table 2A: miRNA sequences used for culture treatments and qPCR

miRBase ID	Name	Sequence
MIMAT0000451	hsa-miR-150-5p	5'-UCUCCCAACCCUUGUACCAGUG-3'
MIMAT0000295	Scramble miRNA (cel-miR-239b-5p)	5'-UUGUACUACACAAAAGUACUG-3'
-	antimiR-150	5'-CACUGGUACAAGGGUUGGGAGA-3'

Table 2B: Primer sequences used for qPCR

Name	Sequence	Orientation	Amplicon size (pb)
28S	5'-GTAGGGACAGTGGGAATCT-3'	<i>forward</i>	108
	5'-CGGGTAAACGGCGGGAGTAA-3'	<i>reverse</i>	
AIFM2	5'-CTGAACGTCCCCTTCATGCT-3'	<i>forward</i>	158
	5'-ATCCCCACTACTAGCCCCTG-3'	<i>reverse</i>	
GAPDH	5'-CGACCACTTTGTCAAGCTCA-3'	<i>forward</i>	228
	5'-AGGGGTCTACATGGCAACTG-3'	<i>reverse</i>	
MYB	5'-TTCCACAGGATGCAGTTCC-3'	<i>forward</i>	208
	5'-TGGGAAGGGGACAGTCTGAA-3'	<i>reverse</i>	
P53	5'-GGCCACTTCACCGTACTAA-3'	<i>forward</i>	156
	5'-GTGGTTTCAAGGCCAGATGT-3'	<i>reverse</i>	
PDCD4	5'-TGTGGAGGAGGTGGATGTGA-3'	<i>forward</i>	249
	5'-TGACTAGCCTTCCCCTCCAA-3'	<i>reverse</i>	

III. DISCUSSION AND SYNTHESIS

The PhD work detailed in this manuscript aimed at better understanding how miRNAs could be involved in the pathophysiology of MG and specifically if they could participate in thymic abnormalities observed in early-onset MG patients and if they can modulate cell function in the thymus and in periphery.

1. Wide analysis of dysregulated miRNAs in the thymus of MG patients

The objective of this study was to investigate the expression of miRNAs in the thymus of early-onset AChR⁺ MG patients. Indeed, as the effector organ of the disease, the thymus displays numerous abnormalities, as detailed in the “State of the Art” part of this manuscript. I investigated dysregulated miRNAs that could be involved in MG and in particular in thymic changes associated with MG.

In general, fold changes of miRNA expression are not elevated and may be very subtle. To make the most of our data and to gain confidence regarding our results, I have used three different but complementary methods to analyze miRNA expression in MG thymuses. We therefore highlighted the involvement of specific dysregulated miRNAs and also of miRNA clusters. In addition, analyzing dysregulated miRNAs and mRNAs together, I also found a new dysregulated inflammatory pathway that could be involved in MG.

We used the Transcriptome Analysis Console (TAC) from Affymetrix, to analyze individually dysregulated thymic miRNAs. We validated miR-7-5p as the most down-regulated miRNA in MG thymuses. We demonstrated that, by targeting CCL21 mRNA, miR-7-5p could be involved in thymic hyperplasia as observed in MG patients. Indeed, CCL21 is upregulated in the thymus of MG patients and is, in part, responsible for the abnormal recruitment of B cells and germinal center development. A question that remains unresolved is why miR-7-5p is down-regulated in the MG thymus. Hansen *et al.* demonstrated that ciRs-7, a circular RNA functioning as a miR-7 sponge, suppresses miR-7 activity, resulting in an increased expression of its targets [111]. I have thus analyzed by qPCR the expression levels of ciRs-7 in the thymus of MG patients compared to controls. However, I did not find any overexpression of ciRs-7 in the thymus of MG patients.

miR-7-5p is transcribed from 3 different loci located on distinct chromosomes (9q21, 15q26 and 19q13). These 3 sequences lead to the production of 3 different pri-miR-7 that will all give an identical mature miR-7-5p. It is not known if miR-7-5p is expressed at a similar level from these 3 different loci and if promoters are identical. Consequently, understanding miR-7-5p regulation at the different promoter level is quite complicated.

Next, we used the webtool MAGIA2 to build integrative analysis and establish correlations between dysregulated mRNAs (extracted from a previous study [259]) and all miRNAs spotted on the chips using matching sequence algorithms. Thanks to this approach, we showed that miR-125a was upregulated in the thymus of MG patients. miR-125a is also involved in systemic lupus erythematosus and Crohn's disease and the authors suggested its role in inflammation [315, 316]. miR-125a modulates indirectly the NF- κ B signaling pathway. Indeed, in an inflammatory context, miR-125a is a regulator of TNFAIP3 (also called A20) [317, 318]. TNFAIP3 is in turn a negative regulator of the NF- κ B signaling pathway, a pathway activated in MG [235].

Here, we demonstrated that miR-125a also regulates another inflammatory pathway by targeting WDR1. WDR1 is associated with inflammation, abnormal T-cell activation and B-cell development [319, 320]. It is also implicated in auto-inflammatory processes associated with IL-18 expression [319]. This observation is of particular interest as IL-18 is overexpressed in the serum of MG patients [321] and the administration of an anti-IL-18 antibody to myasthenic rats leads to the alleviation of the symptoms [322].

Eventually, we used a χ^2 test to evaluate the overrepresentation of miRNAs encoded in specific chromosomal regions. Indeed, with the two previous analysis methods, we had already observed that numerous dysregulated miRNAs belonged to the chromosome X. From our chromosome cluster analysis, we observed the overrepresentation of down-regulated miRNAs encoded in two clusters located at the end of the chromosome X. Among down-regulated miRNAs in these clusters, none of them was known to be involved in MG or AIDs except for miR-20b-3p, which is down-regulated in the

serum of MG patients [285, 289]. miR-20b-3p could act on T_H17 differentiation and more generally may be sensitive to the NF- κ B pathway [323, 324].

These two miRNA clusters are located in the region usually known to be associated with the Fragile X syndrome and surprisingly, patients affected by X-linked disorders tend to develop AIDs more easily [325]. Fragile X patients do not express the protein FMR1 and we observed a decreased expression of *FMR1* mRNA in the thymus of MG patients. Now, the question is to know if there is a link between the regulation of gene expression in this chromosomal region (for mRNA and miRNAs) and autoimmunity. The expression of *FMR1* is very complex and tightly controlled by CGG triplets and the methylation of this region. In addition, other factors regulating chromatin organization are involved, such as CTCF (CCCTC-binding factor) [326]. Consequently, further investigations are needed to assess this question. CTCF is also interestingly involved in the regulation of the expression of several IFN- α genes [327] and investigations related to MG could be conducted. If conclusive, these experiments could deepen the researches regarding epigenetic changes associated with AIDs. Some studies already show the importance of methylation in autoimmunity such as the methylation of the transcription factor AIRE upon estrogen treatment [242], SNP mutations in CTCF binding sites increasing the risk for developing systemic lupus erythematosus [328] or more recently, the methylation of CTLA4 in MG that may promote the occurrence of the disease [329].

In addition, knowing that women are more prone to develop AIDs and EOMG in particular [240, 330], the observation of dysregulated miRNAs on the chromosome X is of particular interest. For example, it would be interesting to compare the difference of miRNAs encoded in this chromosomal region between men and women.

To my knowledge, besides us, only one other team investigated miRNAs in hyperplastic thymuses [314], with some different conclusions.

First, authors do not precise the MG antibody subtype (AChR, MuSK, LRP4), although suggesting AChR⁺ MG patients displaying thymic hyperplasia. As in our study, Li *et al.* do not observe elevated fold changes and observe less than 5% of dysregulated miRNAs among the spotted ones on the chips (1.7%

vs. 4.3% for our study). The authors chose to focus on miR-548k as it is thought to target CXCL13, the B-cell attractant chemokine, involved in the B-cell recruitment in the hyperplastic thymus [216]. However, according to the matching between miR-548k and CXCL13 mRNA, miR-548k shares its seed region with several other miRNAs of the same family such as miR-548b-5p and miR-548c-5p that are also down-regulated in MG thymuses in this study. One can wonder why the authors did not mention this similarity between those miRNAs and why they did not conduct luciferase experiments with these other miRNAs. Moreover, how could we not find the same dysregulated miRNAs? For example, Li *et al.* show that miR-548c-5p is down-regulated (FC=-1.69) and, in our study, the same miRNA is up-regulated in MG thymuses (FC=2.48). Calculations and normalizations are not detailed in the Chinese study, but it is unlikely that such transformations give opposite results. Interestingly, Li *et al.* only considered highly hyperplastic MG thymuses while in our miRnome analysis, we pooled low and high hyperplastic thymuses. After having checked our results, miR-548c-5p is over-expressed in both low and high hyperplastic thymuses, compared to female controls (data not shown). Differences of this importance cannot be explained by different technologies (Affymetrix chips vs. Agilent chips). We can also consider the different populations *i.e.* Chinese vs. French, or at least Asian vs. European. Even if the incidence rate of MG does not differ between Europe and Asia [151], there are more children with juvenile MG in Asia, and HLA associations are different between Asian and European populations. Nonetheless, some miRNAs show similar expression profiles such as miR-148a-5p and miR-487a-5p. Overall, Li *et al.* conclude about the miR-548 family as involved in immunity and autoimmunity and as miR-548k targeting CXCL13 mRNA. Both studies are complementary and both investigate miRNAs susceptible of being involved in thymic hyperplasia.

2. miRNAs and the IFN-I signature characteristic of MG: involvement of the miR-29 family and viral miRNAs

IFN-I was previously investigated in the pathogenesis of MG. Our team showed that Poly(I:C), mimicking a viral dsRNA, is able to induce MG in mice. In addition, the team showed that *in vitro*

Poly(I:C) induces the release of IFN-I from control TECs, especially IFN- β , suggesting that IFN- β is the orchestrator of thymic abnormalities observed in MG [261].

Regarding the miR-29 family, we had a particular interest about miR-29a after the publication of Papadopoulou *et al.* The authors investigated the role of miR-29a in the sensitivity threshold for pathogen infections through the regulation of *Ifnar1*, the IFN-I receptor, in the thymus [308]. Using our miRnome data and then, thanks to RT-PCR validation, I demonstrated that all miR-29 miRNAs family (seven member in total) were down-regulated in the thymus of MG patients.

Knowing that IFN-I, and in particular IFN- β , acts as an orchestrator of thymic changes [261], the aim of our study was to evaluate the sensitivity of miR-29a/b-1 wild-type, heterozygous and knock-out (miR-29 WT/HET/KO) mice, used by Papadopoulou *et al.*, to EAMG.

We demonstrated that miR-29 HET and KO mice displayed a higher sensitivity to the EAMG model compared to CFA-injected mice, to C57BL/6-T-AChR and to miR-29 WT-injected mice.

miR-29 deficiency also leads to higher risk to develop colitis, associated with a T_H17 signature [331]. It is very interesting to note that miR-29 down-regulates *IL23* but no other T_H17 signature genes such as *IL6* or *TGFB*. Knowing that MG is characterized by increased thymic expression of *IL6*, *TGFB* and *IL23* (unpublished results) associated with an increased expression of *IL23R* by thymic T cells [332], it would be interesting to see if there is a link between miR-29 and T_H17 cells in MG. Moreover, miR-29b is a key miRNA for T_H1-mediated inflammation by targeting T-bet and IFN- γ [333]. If miR-29s miRNAs can be linked to T_H1, T_H17 and IFN-I response in MG, they could become major newly-described modulators of AIDs.

In the EAMG model, the thymus does not displays abnormalities as observed in the human disease (see paragraph 2.3.2) [334] but in our experiments, B-cell infiltrates and chemokine overexpression remains to be investigated. Moreover, knowing that Poly(I:C) is able to induce the overexpression of B-cell attractant chemokines, the infiltration of B cells organized in germinal centers and the production of anti-AChR antibodies [260], we will next experiment the "Poly(I:C) model" to miR-29 mice to see if it could potentiate Poly(I:C) effects [262].

It is interesting to note that we observed a “genotype effect”, *i.e.* KO mice are more severely affected than HET mice. This was also observed by van Nieuwenhuijze *et al.* in collagen-induced arthritis [335] where authors suggest a “dose-dependent” effect of miR-29a and miR-29b-1 on mice. However, the deletion of miR-29a/b-1 cluster is rather beneficial on collagen-induced arthritis since B-cell responses and germinal center formation are thought to be altered. Hence, miR-29 KO mice produce less antibodies [335]. If the presence of B cells or germinal center-like structures remains to be investigated in the thymus of miR-29 HET and KO mice, in MG, we do not observe B-cell defects. Smith *et al.* also demonstrated that the deletion of the miR-29a/b1 cluster led to a resistance to develop multiple sclerosis in mice [333] by inducing T-cell apoptosis.

IFN-I is a cytokine produced upon pathogen infections [336]. The miRNA chips that we used for our miRnome study was spotted not only with human miRNA but also 400 viral miRNAs or viromiRs from different species such as Epstein Barr Virus (EBV), Herpes Simplex Virus (HSV) and Rhesus Lymphocryptovirus (RLCV). ViromiRs are miRNAs encoded in the viral genome and expressed through the host’s cellular machinery. Besides taking advantage of the host’s cellular machinery, they can also target host’s genes [337]. We thus investigated if some viromiRs were overrepresented in the thymus of MG patients. Interestingly, only three viromiRs, all from the EBV were found highly expressed in the MG thymus.

Knowing the potential implication of EBV in MG [253], I focused my attention on these EBV miRNAs. EBV encodes 2 miRNAs clusters, BHRF and BART [338]. We demonstrated the overexpression of miR-BART2-5p, miR-BART4-5p and miR-BART16 in the thymus of MG patients. With the help of two Master 1 students that I supervised for their internship, we validated by qPCR the overexpression of miR-BART4-5p. miR-BART16 could not be exploited due to technical problems and miR-BART2-5p was too weakly expressed to be correctly analyzed by qPCR. miR-BART4-5p was not expressed by TECs or thymocytes, as we did not see any variation of the miR-BART4-5p level in these populations between control and MG patients. However, miR-BART4-5p was more expressed in MG patients displaying a high degree of follicular hyperplasia and correlated with the B-cell marker CD19. Further investigations

are now necessary to determine if miR-BART4-5p could affect thymic cell functions and play a role in MG development or chronicity. Regarding the expression of BART miRNAs, the literature is not rich, especially in AIDs, and some investigations are needed. However, in cancerous cell lines, a team showed that miR-BART16 can act on the IFN-I signaling pathway by targeting the CREB-binding protein (CBP), thus preventing a correct promoter activation and inducing an altered IFN-induced gene (ISG) expression [339]. These results sustain that investigations regarding AIDs (and MG in particular), viromiRs and the IFN-I pathway are needed.

An issue raised by these results is the extremely elevated incidence of EBV-positive people among worldwide population. In addition, EBV was previously questioned in other AIDs such as multiple sclerosis and systemic lupus erythematosus [340]. This is where the concept of the mosaic of autoimmunity takes place, where autoimmunity is seen as a puzzle where every arrangement is different and can give rise to different clinical manifestations [341]. Consequently, it requires a specific combination of factors for autoimmunity to declare itself, such as genetic predisposition, stress, hygiene, hormone concentration (vitamin D, estrogen...), increased risk factors (smoking, polluted environment, diet) and infections. In MG, EBV infection does not predispose for autoimmunity but could participate to the triggering or the chronicity of the disease along with other factors.

3. miR-150-5p dysregulation and function in MG

miRNAs, thanks to their accessibility in body fluids and their easy quantification, can be used to monitor disease progression or improvement in the context of AIDs. miR-150 was previously characterized as a biomarker in MG because of its elevated level in the serum of patients and its decrease after thymectomy, accompanied by an improvement of symptoms [108]. After this first published study, our objectives were to understand the causes and consequences of miR-150 increased expression in the serum of MG patients.

I showed that miR-150 expression was increased in the thymus of MG patients and especially in highly hyperplastic thymuses in correlation with the proportion of B cells. We next observed by *in situ* hybridization that miR-150 was preferentially expressed around the GCs, in the mantle zone.

It is not clear what is the exact role of B cells of the mantle zone. They are naïve B cells seemed to be pushed aside during the formation of the GC [342]. They were also reported to be continually transiting through the GC after the recognition of an antigen to make use of an existing GC [343]. However, we did not detect miR-150 inside of the GC. We could hypothesize that the high level of miR-150 induces a low level of its major target MYB involved in cell proliferation, and thus slow down the proliferation of mantle zone B cells [344]. This slow proliferation could slow the resolution process of the GC and participate in its maintenance in the thymus of MG patients. On the contrary, another hypothesis that can be raised is that slow proliferation around the GC could participate in its resolution. A GC is formed after an infection to provide efficient, accurate and extremely precise B-cell reaction. If the proliferation is slowed down, it may be a sign that an intense proliferation is no longer needed. In this case, miR-150 overexpression in MG thymuses would be beneficial and other processes would take place to reinstate GC formation in the thymus.

I also showed that, in periphery, miR-150 was down-regulated in CD4⁺ T cells probably because of a release from activated T cells. We then hypothesized that miR-150 could be either secreted from the thymus or from activated CD4⁺ T cells, or both.

The thymus is able to produce exosomes [345] that could contain and transport miRNAs. However, Skoberg *et al.* showed that the TECs are the cells producing exosomes, and we did not see changes in the expression of miR-150 in TECs. Nevertheless, B cells are also able to secrete exosomes upon stimulation in several compartments [346] and may participate in the miR-150 serum increase in MG patients. In addition, Blanchard *et al.* showed that T cells were able to produce exosomes upon TCR activation [347]. We can observe that many exosomes secretions are made upon activation of the cells; exosomes secretion could be some form of defense mechanism or an alert-related communication process? Indeed, exosomes can be more released under stress conditions [348, 349] and MG thymuses are clearly inflammatory.

Since 2015 and the characterization of miR-150 as a specific biomarker in MG [287], miR-150 has also been described as a biomarker in other inflammatory diseases [350, 351]. miR-150 cannot therefore

be considered as a specific biomarker for MG but rather as a marker of inflammation and immune dysfunction, with a growing biologic interest. Indeed, we showed that serum miR-150 could act on its targets, such as MYB in surrounding cells and therefore, modulate cellular functions. This transcription factor MYB is of particular interest, as it plays key roles in the immune system. In particular, it is an early regulator of T-cell associated diseases and is altered in AIDs [352-354]. In addition, we demonstrated that miR-150 was important regarding cell survival. By treating peripheral lymphocytes with an anti-miR-150, designed to neutralize miR-150, we saw an important cell death of CD4⁺ and CD8⁺ T cells linked to the increased expression of pro-apoptotic genes targeted by miR-150 (Article 3 PhD). In MG, the search for new, targeted and innovative therapies is crucial. One can wonder what role miRNAs could play in such therapies. An anti-miR-150 could be a promising therapeutic agent to counteract the serum increase of miR-150 linked to MG. However, we demonstrated that treating peripheral cells with an anti-miR-150 could affect cell survival. In this context, the effects of anti-miR-150 must be further investigated and in particular which T-cell subset is mostly affected by anti-miR-150-induced apoptosis. Indeed, if anti-miR-150 induced more specifically the death of T_H1 or T_H17 cells, it could therefore have an interesting anti-inflammatory effect for various AIDs. Any therapy aiming at decreasing miR-150 level in the serum would not necessarily restore an apoptosis process that was down-regulated upon miR-150 increase.

Regarding the biomarker function of miRNAs, as mentioned in the study of miR-150 and in the paragraph 1.3.5.1 of the "State of the Art" part, it remains difficult to have a clear opinion. The literature is extremely rich about miRNAs as biomarkers and especially in cancer. To me, it is difficult to consider miRNAs as a diagnosis biomarkers. Other tools are much more powerful to help in the diagnosis *i.e.* antibody dosage for AIDs, imagery... However, they can be of precious help in the case of difficult diagnosis, in association with known dysregulated protein, such as in pancreatic cancer [355]. In this study, the authors combined protein markers identified in cell culture supernatant's exosomes and dysregulated miRNAs identified by microarray. Combination of protein and miRNAs improved the

sensitivity and specificity to detect malignancies. miRNAs can nonetheless be interesting prognosis markers and be used for treatment monitoring.

4. Conclusion

When I arrived in the lab, this Master 2 and then PhD project was quite innovative. Indeed, miRNA tools were just developing, literature was not very rich regarding miRNAs in AIDs, inflammation and rare diseases, and was more focused about miRNA structure and biogenesis. I had to face many technical issues regarding miRNA analyses, RT-PCR, normalizers... However, my efforts were not done in vain and led to significant findings described in three first-author publications. I was also involved in two other articles of the team and in a review. I also had the rewarding opportunity to manage four Master 1 students: Nolwenn Samson, who investigated miR-125a-5p expression in the thymus and its link with miR-29 and TNFAIP3, Fabien Delisle, who worked on the miR-7 family, and Melvyn Wothor and Indusha Kugathas who conducted experiments on EBV miRNAs.

Altogether, the studies described in this manuscript allowed us to open new research avenues, to better understand the involvement of miRNAs in AIDs and in particular in MG, and to investigate molecular changes in the thymus, the MG effector organ. The objectives of this PhD were fulfilled. There is still a lot to do regarding the involvement of EBV in MG and the hypothesis of feminine predisposition, chromosome X and MG. These research avenues will be exploited in the near future.

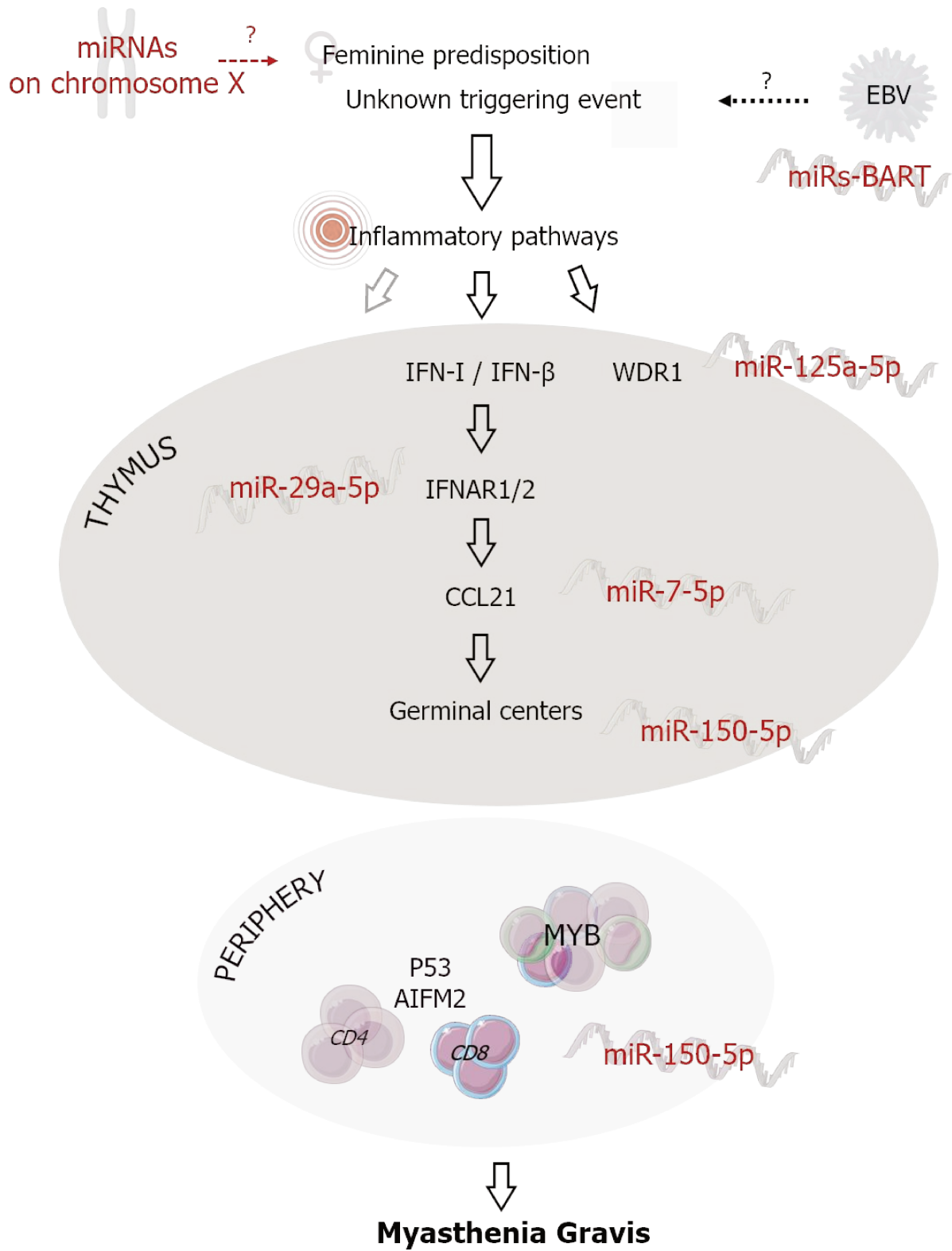


Figure 13: Summary diagram of investigated miRNAs during this PhD

IV. BIBLIOGRAPHY

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V. ANNEXES

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Issue: *Myasthenia Gravis and Related Disorders*

REVIEW

Thymus involvement in early-onset myasthenia gravisMélanie A. Cron,^{1,2,3} Solène Maillard,^{1,2,3} José Villegas,^{1,2,3} Frédérique Truffaut,^{1,2,3}
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It has long been established that the thymus plays a central role in autoimmune myasthenia gravis (MG) because of either thymoma or thymic hyperplasia of lymphoproliferative origin. In this review, we discuss thymic changes associated with thymic hyperplasia and their implications in the development of an autoimmune response against the acetylcholine receptor (AChR). The hyperplastic MG thymus displays all the characteristics of tertiary lymphoid organs (TLOs): neoangiogenic processes with high endothelial venule and lymphatic vessel development, chemokine overexpression favoring peripheral cell recruitment, and ectopic germinal center development. As thymic epithelial cells or myoid cells express AChR, a specific antigen presentation can easily occur within the thymus in the presence of recruited peripheral cells, such as B cells and T follicular helper cells. How the thymus turns into a TLO is not known, but local inflammation seems mandatory. Interferon (IFN)- β is overexpressed in MG thymus and could orchestrate thymic changes associated with MG. Knowledge about how IFN- β is induced in MG thymus and why its expression is sustained even long after disease onset would be of interest in the future to better understand the etiological and physiopathological mechanisms involved in autoimmune MG.

Keywords: germinal centers; chemokines; interferon type-I; pathogen infection; miRNAs

Introduction

Myasthenia gravis (MG) with anti-acetylcholine receptor (AChR) antibodies is characterized by muscle weakness and fatigability. The disease generally begins with ocular symptoms (ptosis and/or diplopia) and extends to other muscles in 80% of cases. It is a prototype autoimmune disease in which the target organ, the muscle, is distinct from the effector organ, the thymus. In MG patients with anti-AChR antibodies, functional and morphological abnormalities of the thymus are frequently observed. Patients can display a thymoma, especially after 50 years old, or B cell infiltrations associated with thymic hyperplasia of lymphoproliferative origin in younger patients and mainly women.¹

In contrast, no thymic abnormalities are observed in MG with muscle-specific kinase antibodies. In MG with LRP4 antibodies, thymic hyperplasia of lymphoproliferative origin has also been observed in a few patients but not further investigated so far.² Here, we mainly focus on thymic changes occur-

ring in early-onset AChR⁺ MG patients occurring usually before 45–50 year of age. Most of these MG patients present high level of anti-AChR antibodies and thymic follicular hyperplasia. Sex hormones may play a role in this form of the disease, as more than 80% of patients with follicular hyperplasia are women.

The normal thymus

The thymus is a primary lymphoid organ that provides a complex environment essential for T cell maturation and differentiation during their migration within the cortical and medullary thymic compartments. This is orchestrated via interactions between T cells and mainly thymic epithelial cells (TECs), but also other stromal cells, such as dendritic cells, fibroblasts, and myoid cells.^{3,4} In the cortex, in their first differentiation steps, immature T cells become progressively double positive for CD4 and CD8 coreceptors and acquire a complete T cell receptor (TCR). Further successful differentiation

depends on the quality and the specificity of TCR interaction with major histocompatibility complex (MHC) on stromal cells. A large majority of thymocytes are eliminated because the TCR–MHC interaction is too weak (death by neglect), and only a few thymocytes successfully pass positive selection. In contrast, in the medulla, thymocytes are eliminated if TCR–MHC interactions are too strong (negative selection). This is the basis of the central tolerance process based on the ability of TECs to express a repertoire of tissue-specific antigens (TSAs) that are presented to T cells. The expression of these TSAs is monitored by the autoimmune regulator AIRE or the transcription factor FEZ family zinc finger 2.^{5,6} In this context, TECs are able to express the different AChR subunits whose expression is controlled by AIRE.^{7,8} Lately, AIRE expression has been demonstrated to be downregulated by estrogen, explaining the female predisposition to autoimmunity, including MG, as detailed in this special issue by Berrih-Aknin *et al.*⁹ Thymic myoid cells that possess the antigenic characteristics of skeletal muscle cells also express all AChR subunits and display a functional AChR.^{7,10}

TECs are also involved in the selective induction of natural regulatory T cells.¹¹ Medullary TECs promote the generation of regulatory T cells and favor their functionality.¹² Hassall's corpuscles are also observed in thymic medulla and are formed by concentrically arranged TECs that could correspond to highly differentiated TECs. Their precise function remains unclear.¹³ Altogether, this highlights that the thymus is a complex organ indispensable to set immune central tolerance, and thymic dysfunction can lead to autoimmunity.

Thymic abnormalities in early-onset MG

Pathological alterations of the thymus are very often observed in AChR-MG patients with a generalized disease. Thymic hyperplasia of lymphoproliferative origin is observed in 50–60% of these patients, a thymoma is detected in approximately 15% of the patients, and, in the other cases, the thymus is atrophic or involuted with mainly adipose tissue and residual areas of thymic parenchyma. As described below, the hyperplastic thymus in MG displays numerous features normally observed in secondary lymphoid organs (SLOs) and, owing to its inflammatory status, the hyperplastic MG thy-

mus is even considered a tertiary lymphoid organ (TLO).

Abnormal T cell functionality

In the thymus of MG patients, no obvious changes are observed concerning the frequency of CD4⁺ and CD8⁺ T cells that are exported to the periphery.¹⁴ However, other changes have been demonstrated. Natural regulatory T cells that differentiate in the thymus are clearly less functional in the thymus of MG patients, and this is also observed to a lesser degree in regulatory T cells in the periphery.^{15,16} Later, it was demonstrated that the altered immune regulatory function observed for T cells in MG patients was not only linked to the functional defect of regulatory T cells. Indeed, effector T cells from the thymus of MG patients are also resistant to suppression by regulatory T cells, and this is probably due to the inflammatory thymic environment.¹⁷ Immunoregulatory defects are thus observed in both regulatory and effector T cells in MG patients. This is associated with changes in the expression of proinflammatory cytokines by MG T cells, such as an interleukin (IL)-17 signature in regulatory T cells and increases in interferon (IFN)- γ , IL-21, and tumor necrosis factor α expression in both regulatory and effector T cells.¹⁷ These data suggest that the inflammatory milieu of the MG thymus alters the function and plasticity of CD4⁺ T cells, leading to impaired function of regulatory T cells and resistance of effector T cells to suppression.

Peripheral cell infiltrations leading to germinal center development

B cells can be detected at low levels (around 0.1–0.5% of thymocytes) in normal thymuses, and they are located mainly in the medulla and perivascular spaces.¹⁸ Their precise role is not clear, but medullary B cells could be involved in negative selection, while perivascular spaces contain mainly plasma cells that could confer protection against pathogens.^{19,20}

One of the main features characterizing the thymus in AChR⁺ MG is the presence of an increased number of B cells, often organized in germinal centers (GCs). GCs can sometimes be observed in normal thymuses, increasing with age,^{21,22} but this is particularly a characteristic of AChR⁺ MG thymuses (Fig. 1A).²³ The presence of thymic GCs in other autoimmune diseases has not been clearly established. There is a clear association between the

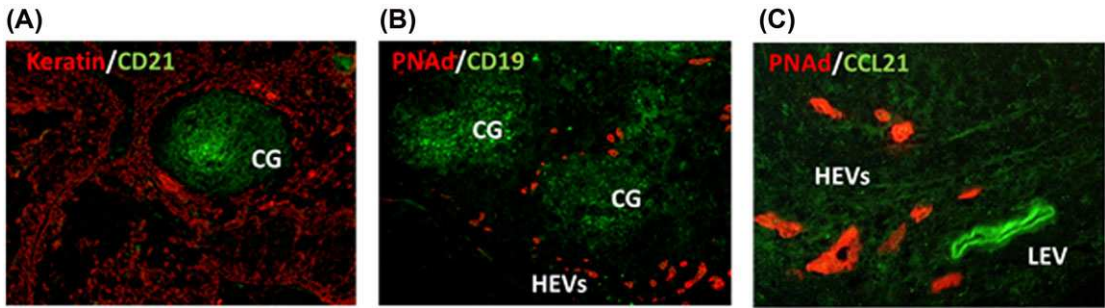


Figure 1. Thymic changes in MG patients. Thymic sections from MG patients were labeled (A) in red with an anti-keratin antibody and in green with an anti-CD21 antibody for B cells and follicular dendritic cells to localize GCs; (B) in red for HEVs with an anti-peripheral node addressin (PNAd) antibody and a biotinylated anti-rat IgM followed by an Alexa-Fluor 594 streptavidin and in green with an anti-CD19 antibody for B cells; (C) in red for HEVs and in green for CCL21 with an anti-human CCL21 followed by an Alexa-Fluor 488. CG, *centre germinatif* (germinal center). Reprinted with permission from Ref. 36.

thymic follicular development and the age and gender of the patients: (1) the youngest patients display the highest degree (with three or more GCs per thymic section) and the older patients display the lowest degree (with fewer than two GCs per thymic section) of follicular hyperplasia, and (2) 80% of patients with thymic hyperplasia are women.¹ Recently, an increased number of T follicular helper (T_{FH}) cells have also been described in the periphery and in the thymus of MG patients.²⁴ T_{FH} cells are normally located in the GCs of SLOs, where they play a central role in B cell maturation and antibody production. The MG thymus contains all components to set an immune response as in SLOs and in particular an immune sensitization against AChR: (1) medullary TECs and myoid cells express AChR,^{7,10} (2) thymic B cells can produce anti-AChR antibodies,^{25,26} and (3) anti-AChR autoreactive T cells are present.²⁷ The thymuses of AChR⁺ patients have been shown to contain B cells producing anti-AChR antibodies, suggesting a possible expansion of specific B cells.^{28,29} However, the polyclonality of thymic B cells and an overall increased expression of immunoglobulin genes, independent of antigenic specificity, have been demonstrated in MG patients.^{29–31} The clear implication of the thymus in MG is also demonstrated using immunodeficient mice that are grafted with thymic biopsies from MG patients. Indeed, almost all the animals display human anti-AChR Abs in their serum, and 50% of them develop MG-like symptoms in correlation with the loss of AChR at the muscle endplates.³²

As described by Truffault *et al.*, AChR⁺ MG patients with thymic hyperplasia have higher anti-AChR antibody titers than patients with thymoma or involuted thymuses, and a clear correlation exists between the degree of thymic hyperplasia and serum levels of anti-AChR antibodies.¹ Moreover, the number of GCs is reduced in patients undergoing corticosteroid treatment.²³ All of these observations support the role of the thymus in the pathogenesis of MG, and thymectomy is often advised for AChR⁺ MG patients. A recent randomized trial in MG patients treated with prednisolone demonstrated the benefit of thymectomy in improving MG symptoms over a 3-year follow-up period.³³

Neoangiogenic processes

The development of thymic hyperplasia is supported by active neoangiogenic processes with high endothelial venule (HEV) and lymphatic endothelial vessel (LEV) development. HEVs are found in SLOs and chronically inflamed tissues. They are specialized venules bearing on their luminal surface diverse chemokines and expressing high levels of peripheral node addressin carbohydrate, which allows the homing of lymphocytes and dendritic cells.³⁴ Using immunohistochemistry, only a few HEVs are detected in the thymus of non-MG adults.³⁵ In contrast, in the thymus of MG patients, increased numbers of HEVs are observed around GCs (Fig. 1B), correlating with the degree of thymic hyperplasia. Such high numbers of HEVs in hyperplastic thymuses suggest that peripheral cells enter

Table 1. List of the chemokines that are abnormally expressed in the thymus of early-onset MG patients

Chemokine	Receptor(s)	Expression in MG thymuses
CCL17	CCR4	Increased expression in Hassall's corpuscle and surrounding cells ⁴² Expressed on HEVs in MG thymus ⁴²
CCL19	CCR7	Upregulated in MG ^{31,36}
CCL21	CCR7	Upregulated in MG thymus ^{31,36}
CCL22	CCR4	Increased expression in Hassall's corpuscle and surrounding cells ⁴²
CXCL9	CXCR3	Upregulated in MG thymus ³¹
CXCL10	CXCR3	Upregulated in MG thymus ⁴⁰
CXCL11	CXCR2/CXCR7	Upregulated in MG thymus ³¹
CXCL12	CXCR4/CXCR7	Expressed on HEVs in MG thymus ³⁵
CXCL13	CXCR5	Upregulated in MG thymus ^{23,31}
RANTES/CCL5	CCR1/CCR3/CCR5	Overexpressed in thymic epithelial cells in MG ⁴¹

the MG thymus through these specialized vessels. The number of thymic HEVs is reduced in patients undergoing corticosteroid treatment.³⁵

The increased expression of lymphatic markers, such as vascular endothelial growth factor receptor 3 and PROX1, has been demonstrated in hyperplastic thymuses, also suggesting the expansion of the lymphatic system. LEVs expressing specifically CCL21 have been described in hyperplastic MG thymuses, as detailed below (Fig. 1C).³⁶ Lymphangiogenesis occurs throughout life in homeostasis and diseases. It has been described in lymph nodes after immunization, where it was shown to be dependent on the entry of B cells.³⁷

Efficient cell recruitment via HEVs or LEVs is a multistep process, and chemokines displayed on vessels are involved in the transmigration of circulating cells.³⁸

Chemokine overexpression

Chemokines play a central role in thymopoiesis through their chemotactic and chemorepulsive properties, allowing for the recruitment of prothymocytes, the migration of thymocytes from the cortex to the medullary region, and their export to the periphery. Chemokines are also crucial for peripheral cell recruitment in SLOs.³⁹ A transcriptome study demonstrated that thymic chemokine expression profiles differ in MG patients in association with increased chemotactic properties of hyperplastic thymic extracts.³¹ As described below, several chemokines are increased or abnormally expressed in the MG thymus, as detailed in Table 1.^{23,31,35,36,40–42} Altogether, these data show that chemokine profiles are strongly modified in

the MG thymus. They are overexpressed in different cells and probably play a central role in peripheral cell recruitment in the MG thymus and the development of ectopic GCs.

Expression of CXCL12 and CCL17 by ectopic high endothelial venules. Under physiological conditions, diverse chemokines are displayed on HEVs in SLOs and are involved in the transmigration of circulating cells across HEVs.³⁸ The expression of several chemokines on thymic HEVs was investigated by Weiss *et al.* Chemokines investigated were known to be expressed by HEVs in SLOs or chronically inflamed tissues and also known to be dysregulated in the MG thymus at that time: CCL19, CCL21, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, and RANTES/CCL5.³⁵ Among these chemokines, only CXCL12 was found expressed on the lumen side of thymic HEVs. Moreover, antigen-presenting cells, such as monocytes/macrophages, dendritic cells, and B cells expressing CXCL12 receptor CXCR4, are detected inside and around thymic HEVs. CCL17 has also been described on thymic HEVs and could favor the recruitment of CCR4⁺ dendritic cells.⁴²

Abnormal expression of CCL21 on LEVs. In the periphery, CCL21 is known to play a central role in immune surveillance and defense by controlling the circulation of T cells and dendritic cells within lymphoid and peripheral organs. CCL21 is also involved in naive B cell recruitment.³⁶ In the thymus, CCL21 and CCL19, both interacting with the receptor CCR7, play important roles in thymopoiesis.⁴³ In MG patients, thymic hyperplasia is specifically associated with the thymic overexpression of CCL21 and CCL19. The overexpression

of CCL21 in hyperplastic MG thymuses is due to LEVs (Fig. 1C). Thymic overexpression of CCL21 in MG could thus play a role in bringing naive B cells, but perhaps also peripheral dendritic cells and T cells, in contact with the inflammatory environment characteristic of MG thymus, where they can be sensitized against AChR.^{36,44}

Overexpression of CXCL13 by thymic epithelial cells. CXCL13 is the most potent chemoattractant for B cells. CXCL13 interacts with cells through its receptor CXCR5, which is also expressed on T_{FH} cells. In SLOs, CXCL13 participates in GC formation and it is also overexpressed at inflammatory sites characterized by ectopic GC development.⁴⁵ CXCL13 mRNA is only expressed at very low levels in normal thymuses. However, in AChR⁺ MG patients, thymic CXCL13 expression is strongly increased. Even if CXCL13 is known to be produced by GCs, medullary TECs in MG patients also expressed abnormal levels of CXCL13.²³ The active recruitment of peripheral B cells but also T_{FH} cells via CXCL13 in MG thymuses could support the development of ectopic GCs.

Inflammation is mandatory to reveal properties of CXCL13. As CXCL13 is overexpressed by medullary TECs in MG patients,²³ transgenic mice overexpressing CXCL13 under the control of the keratin 5 (*K5*) promoter were developed. The objective was to mimic thymic overexpression of CXCL13 by medullary TECs as in the MG thymus. Data demonstrate that transgenic *K5-Cxcl13* mice overexpress CXCL13 in their thymus, but this does not induce the recruitment of B cells. However, in inflammatory conditions induced by the injections of a molecule mimicking dsRNA from viral infection (polyinosinic–polycytidylic acid (poly(I:C))) or the immunization with a strong adjuvant, the recruitment of B cells is detected in the thymus.⁴⁶ The classical mouse model of MG is induced by immunizing animals with purified AChR extracted from the electric organ of torpedo fish (T-AChR) together with complete Freund's adjuvant. If the animals produce antibodies that induce loss of AChR at the muscle endplate and dysfunction of the neuromuscular transmission, this model does not present thymic abnormalities.⁴⁷ Using the *K5-Cxcl13* mice, it was demonstrated that mice are more susceptible to experimental autoimmune MG, with stronger clinical signs, higher titers of anti-AChR antibodies,

increased thymic B cells, and the development of GC-like structures in the thymus.⁴⁶

Altogether, these data suggest that thymic inflammation is mandatory to reveal the chemotactic properties of CXCL13. Inflammation subsequent to pathogen infection appears to be a key event to optimize the recruitment of mature lymphocytes to peripheral organs and even the thymus.^{48,49} IFN- γ that is released during pathogen infection could favor cell motility.⁵⁰

Pathogen infection signature associated with Toll-like receptor expression

Pathogens are major environmental factor candidates for driving/perpetuating autoimmunity. However, since autoimmunity onset can occur well after a possible triggering infection, when the pathogen might have been cleared or the antiviral immune responses might have subsided, it is difficult to link infections with autoimmune diseases.

Nevertheless, the presence of poliovirus-infected macrophages and Epstein–Barr virus (EBV)-infected B cells has been shown in MG thymus.^{51,52} In MG, striking evidence of chronic inflammation and emerging data on persistent viral infections in the thymus of MG patients strongly support the hypothesis that the innate immune system may promote, exacerbate, and/or maintain the autoimmune condition.

Toll-like receptors (TLRs) play major roles in innate immunity. TLRs 1–10 recognize specific microbial-derived molecular structures.⁵³ The expression levels of TLRs in the thymus of MG patients have been analyzed in different studies reviewed by Robinet *et al.*⁵⁴ Briefly, TLRs 1–9 are all expressed in control thymuses. In MG thymuses, the overexpression is described for TLR3, TLR4, TLR6, TLR7, TLR8, and TLR9, and correlations between CD19 (a B cell marker) mRNA expression and TLR6, TLR8, and TLR9 mRNA expression have been demonstrated.⁵⁴

To investigate the potential consequences of pathogen infection on the thymus, the effects of TLR agonists have been analyzed *in vitro* in human cultured TECs and *in vivo* in mouse thymus. In human TECs, among all TLR agonists, poly(I:C), a synthetic analog of dsRNA mimicking viral infections, specifically induces the thymic overexpression of α -AChR, but not other AChR subunits or TSAs. This induction is mediated by the release of IFN- β and

is completely inhibited by IFN-I receptor or IFN- β blocking antibodies.⁵⁵ In C57Bl/6 mice, injections of poly(I:C) for 1 week also trigger the specific thymic expression of α -AChR. Poly(I:C) injections also induce other thymic changes, such as the overexpression of IFN-I subtypes, CXCL13, and CCL21, associated with an increased recruitment of thymic B cells. All of these thymic changes disappear after 1 week, but prolonged poly(I:C) injections over 6–8 weeks induce the development of an anti-AChR response in the periphery, with the proliferation of autoreactive B cells against AChR in lymph nodes and the production of anti-AChR antibodies. The presence of circulating anti-AChR antibodies leads to MG symptoms with a loss of AChR on the muscle diaphragm and muscle weakness.⁵⁵

In accordance with these observations on poly(I:C) effects, dsRNA-signaling pathways are activated in MG thymuses.⁵⁵ This could be related to the thymic EBV signature observed in MG, as EBV encodes small RNAs that trigger TLR3 signaling and induce IFN-I and proinflammatory cytokine expression, similar to poly(I:C).^{52,56}

Central role of interferon- β

Inflammation constitutes an essential component of the innate immune response induced by pathogens that stimulate the release of proinflammatory molecules. IFN-Is are secreted by various cells as an antiviral defense mechanism and, depending on the context, can either be considered anti-inflammatory, as in multiple sclerosis, or proinflammatory, as in systemic lupus erythematosus.^{57–59} The implication of IFN-I in MG has long been suggested in various ways: (1) clinical reports demonstrate the development of MG after IFN- α or IFN- β therapies,⁶⁰ (2) antibodies against IFN- α are found in some MG patients, mainly those with thymoma,⁶¹ and (3) IFN- β is overexpressed in MG thymuses together with numerous IFN-I-induced genes, as detailed by Poëa-Guyon *et al.*^{55,62,63}

IFN-I proteins, specifically IFN- β , induce the specific expression of α -AChR by TECs and not that of other TSAs. It also increases TEC death and the uptake by dendritic cells of TEC proteins, potentially the α -AChR that is overexpressed in IFN- β -treated TECs. In parallel, IFN- β triggers the expression of CXCL13 and CCL21 by TECs and lymphatic endothelial cells, respectively, and consequently could favor peripheral cell recruitment

in the thymus. IFN- β also induces the expression of B cell activating factor (BAFF), which favors B cell survival and is overexpressed by TECs in MG thymus.⁶⁴ Similar changes are observed *in vivo*, as the injections of poly(I:C) to C57Bl/6 mice trigger a thymic overexpression of IFN- β and IFN- α 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 gene knockout mice injected with poly(I:C).⁶⁴

Altogether, these observations demonstrate that IFN- β orchestrates thymic events that could lead to MG by triggering the overexpression of α -AChR, probably inducing thymic dendritic cell auto-sensitization, the abnormal recruitment of peripheral cells, and GC formation. All of these observations are summarized in Figure 2. It was also demonstrated that IFN-I subtypes might play central roles in thymoma-associated MG. Huge increases of IFN-I subtypes are observed in thymoma-associated MG, but not in thymomas without MG or in control thymuses. These results reinforce a specific role of IFN-I in the anti-AChR response associated with MG.⁶⁵

In MG patients, the fact that IFN- β is overexpressed even long after disease onset suggests a persistent induction owing to the presence of a pathogenic agent, as discussed above with EBV, or an altered retrocontrol mechanism to repress IFN-I signaling that could affect downregulation of IFN-I receptor from the cell surface, dephosphorylation of signaling components of the IFN-I pathway, induction of negative regulators, and induction of microRNAs (miRNAs).

Dysregulated expression of miRNAs

MiRNAs are small RNAs that are posttranscriptional regulators of gene expression. They interact specifically with mRNAs, leading to their degradation or to the inhibition of their translation and consequently decreasing protein expression. Recent studies have investigated and identified circulating miRNAs as readily accessible blood biomarkers for MG patients.^{66–68} The differential expression of some miRNAs is also observed in peripheral blood mononuclear cells from MG patients.^{69,70}

As miRNAs play an important role in the regulation of inflammation and autoimmunity, we can envisage that altered miRNA expression could be involved in thymic changes associated with MG.

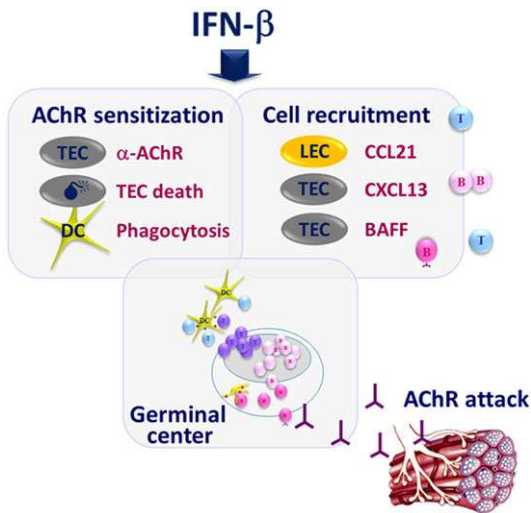


Figure 2. Central role of IFN- β in thymic changes associated with MG. IFN- β induces the expression in thymic epithelial cells (TECs) of α -AChR, the main autoantigen in MG. This effect is very specific to α -AChR, as IFN- β does not induce the expression of other AChR subunits or other tissue-specific antigens. IFN- β also induces TEC death and the uptake of TEC proteins by dendritic cells, suggesting a potential sensitization of dendritic cells to α -AChR. In parallel, IFN- β increases the expression of the chemokines CXCL13 and CCL21 by TECs and lymphatic endothelial cells, respectively. These two chemokines are involved in GC development and are overexpressed in MG with follicular hyperplasia. We also demonstrated that the B cell activating factor (BAFF), which favors autoreactive B cells, was overexpressed by TECs in MG thymus and was also induced by IFN- β . Altogether, these results demonstrate that IFN- β plays a central role in thymic events leading to MG by triggering the overexpression of α -AChR, probably leading to auto-sensitization of thymic dendritic cells against AChR and the abnormal recruitment of peripheral cells involved in GC formation. Reprinted with permission from Ref. 54.

Besides, as miRNAs are known to be expressed in the thymus and exported in exosomes to improve cellular communications,⁷¹ it would be interesting to investigate the modifications of thymic miRNA expression in a myasthenic context. Environmental factors, such as infections, are able to modify the expression of specific miRNAs and eventually alter thymic function.⁷² Moreover, miR-29a is an important regulator of the IFN-I signaling pathway, targeting IFN-I receptor in TECs and reducing cell sensitivity to IFN-I and consequently pathogen infections.⁷³ MiR-205 has also been demonstrated to be important in maintaining thymopoiesis upon inflammatory perturbations.⁷⁴ All of these studies highlight the role of miRNAs in thymus homeostasis

in mice and suggest that altered miRNA expression in the human thymus could be observed in MG patients.

Conclusions

Chronically inflamed tissues can turn into TLOs that possess numerous specific characteristics of SLOs, such as the development of a vascular system, the infiltration of leukocytes, and the presence of GCs, sustained by the overexpression of chemokines and inflammatory cytokines. The hyperplastic MG thymus displays all the characteristics of TLOs. Recent data suggest that IFN- β could initiate and orchestrate these thymic changes and the intrathymic autoimmune response to AChR. Future investigations are needed to decipher the upstream events, what is triggering thymic inflammation, and why this inflammation is sustained over time.

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Competing interests

The authors have declared no competing interests.

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Use of Toll-Like Receptor Agonists to Induce Ectopic Lymphoid Structures in Myasthenia Gravis Mouse Models

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Myasthenia gravis (MG) is an autoimmune disease mediated by autoantibodies against the acetylcholine receptor (AChR) at the neuromuscular junction. MG symptoms are characterized by muscle weaknesses. The thymus of MG patients is very often abnormal and possesses all the characteristics of tertiary lymphoid organs such as neoangiogenesis processes, overexpression of inflammatory cytokines and chemokines, and infiltration of B lymphocytes leading to ectopic germinal center (GC) development. We previously demonstrated that injections of mice with polyinosinic-polycytidylic acid [Poly(I:C)], a synthetic double-stranded RNA mimicking viral infection, induce thymic changes and trigger MG symptoms. Upon Poly(I:C) injections, we observed increased thymic expressions of α -AChR, interferon- β and chemokines such as CXCL13 and CCL21 leading to B-cell recruitment. However, these changes were only transient. In order to develop an experimental MG model associated with thymic GCs, we used Poly(I:C) in the classical experimental autoimmune MG model induced by immunizations with purified AChR emulsified in complete Freund's adjuvant. We observed that Poly(I:C) strongly favored the development of MG as almost all mice displayed MG symptoms. Nevertheless, we did not observe any ectopic GC development. We next challenged mice with Poly(I:C) together with other toll-like receptor (TLR) agonists known to be involved in GC development and that are overexpressed in MG thymuses. Imiquimod and CpG oligodeoxynucleotides that activate TLR7 and TLR9, respectively, did not induce thymic changes. In contrast, lipopolysaccharide that activates TLR4 potentiated Poly(I:C) effects and induced a significant expression of CXCL13 mRNA in the thymus associated with a higher recruitment of B cells that induced over time thymic B-lymphoid structures. Altogether, these data suggest that tertiary lymphoid genesis in MG thymus could result from a combined activation of TLR signaling pathways.

Keywords: toll-like receptor, lipopolysaccharide, poly(I:C), autoimmunity, B cells, myasthenia gravis, germinal centers

Abbreviations: AChR, acetylcholine receptor; CFA, complete Freund's adjuvant; EAMG, experimental autoimmune myasthenia gravis; GC, germinal center; IFN, interferon; Ig, immunoglobulin; KO, knockout; LPS, lipopolysaccharide; MG, myasthenia gravis; poly(I:C), polyinosinic-polycytidylic acid; SLO, secondary lymphoid organ; TEC, thymic epithelial cell; TLO, tertiary lymphoid organ; TLR, toll-like receptor.

INTRODUCTION

Secondary lymphoid organs (SLOs) such as lymph nodes, spleen, or tonsils are environment specialized for the interaction between lymphocytes and antigen-presenting cells in order to develop an adaptive immune response. SLOs provide the optimized microenvironment for germinal center (GC) reactions. When T and B cells enter into SLOs *via* the vascular network, they can encounter primed antigen-presenting cells engaging a GC reaction. Within GCs, B cells undergo clonal expansion, immunoglobulin (Ig) class switch, and somatic hypermutation leading to the development of B cells expressing high-affinity antibodies that differentiate into antibody-secreting plasma cells and memory B cells in order to mediate a sustained protection against invading pathogens. Chronic inflamed tissues can turn into tertiary lymphoid organs (TLOs) associated with ectopic GC reactions. These tissues are characterized by the development of a vascular system, the infiltration of leukocytes, the presence of GCs, sustained by the overexpression of chemokines and inflammatory cytokines (1). TLOs are observed in many organ-specific autoimmune diseases such as in the thymus in myasthenia gravis (MG), the salivary glands in Sjogren's syndrome, the thyroid gland in Graves' disease and Hashimoto's thyroiditis, and the cerebral meninges in multiple sclerosis (2, 3). The common feature for all these diseases is that tertiary lymphoid neogenesis occurs in tissues harboring the autoantigen.

Myasthenia gravis with anti-acetylcholine receptor (AChR) antibodies is characterized by muscle weakness and fatigability. MG is a prototype autoimmune disease in which the target organ, the muscle, is distinct from the effector organ, the thymus. In MG patients with anti-AChR antibodies, functional and morphological abnormalities of the thymus are frequently observed: either a thymoma or B-cell infiltrations with more than 75% of patients exhibiting thymic hyperplasia of lymphoproliferative origin with ectopic GC development (4). There is a clear relationship between the degree of hyperplasia and the serum level of anti-AChR antibodies (4), and a recent randomized clinical trial has clearly demonstrated that thymectomy improved clinical outcomes (5). Moreover, immunodeficient mice engrafted with human MG thymic tissue have anti-AChR antibodies in the serum and animals displayed MG-like symptoms that correlated with the loss of AChR at the muscle endplates (6). Altogether these data demonstrate that the thymus is clearly involved in the MG. The hyperplastic MG thymus displays all the characteristics of TLOs (7): neoangiogenic processes with high endothelial venules (HEVs) and lymphatic vessels development (2, 8, 9), chemokine overexpression (such as CXCL13 and CCL21) favoring peripheral-cell recruitment (8, 10, 11), and ectopic GC development (4). Moreover, the autoantigen (α -AChR) involved in MG is directly expressed in the thymus by thymic epithelial cells (TECs) and myoid cells (12). Within thymic GCs, interactions have been described between T follicular helper cells and B cells known to induce B-cell maturation and antibody production in the SLOs (13). The presence of anti-AChR autoreactive T cells (14) and B cells producing anti-AChR antibodies (15, 16) has also been described in the thymus of MG patients. The exact mechanisms initiating these thymic changes and the intrathymic autoimmune

response to AChR are not yet clearly defined but local inflammation seems to be mandatory. The overexpression of interferon (IFN)- β and IFN-I-induced genes has been observed in the MG thymus even long after the disease onset (17, 18). Later on, our team demonstrated that IFN- β could be the orchestrator of thymic changes associated with MG. Indeed, IFN- β induces specifically α -AChR expression in TECs. IFN- β also increases TEC death and the uptake of TEC proteins by dendritic cells, suggesting a role in α -AChR sensitization. In parallel, IFN- β increases the expression of the chemokines CXCL13 and CCL21 by TECs and lymphatic endothelial cells, respectively. These two chemokines are involved in GC development and are overexpressed in MG thymus with follicular hyperplasia. We also demonstrated that the B-cell activating factor, which favors autoreactive B-cells, was overexpressed by TECs in MG thymus and was induced by IFN- β in TEC cultures (19).

Myasthenia gravis, as other autoimmune diseases, is a multifactorial disease involving genetic susceptibility, hormonal influence (20) or environmental factors including pathogen infections (21). Persistent viral infections in specific organ are suspected to favor tertiary lymphoid genesis associated with autoimmunity (22). The thymus of MG patients is characterized by increased expression of toll-like receptors (TLRs) involved in pathogen recognition, such as TLR3, TLR4, TLR7, and TLR9, but also by increased expression of IFN- β and numerous IFN-induced genes (17, 18, 23–26). All these data suggest that MG could be triggered by pathogen infections.

A well-defined experimental autoimmune MG (EAMG) model is induced by immunizing wild-type mice with a mixture of complete Freund's adjuvant (CFA) and AChR purified from *Torpedo californica* fish (27). In this model, mice develop antibodies against T-AChR which target AChR on muscle cells. Even though the EAMG model is relevant to study muscle weakness caused by the anti-AChR antibody attack, it does not completely recapitulate the human disease, as the thymus does not display ectopic GC development (28). Using a transgenic mouse line with thymic overexpression of CXCL13, we clearly demonstrated that inflammation is mandatory to induce B-cell recruitment and GC development in the EAMG model (29). Using polyinosine-polycytidylic acid [Poly(I:C)], a synthetic analog of double-stranded (ds)RNA mimicking viral infection, we demonstrated that repeated injections of C57BL/6 mice rapidly induce thymic changes similar to what could happen in MG patients. Briefly, after 1 week of injections, we observed thymic induction of IFN- β associated with an overexpression of CXCL13 and CCL21, leading to transient thymic B-cell recruitment. In parallel, we also demonstrated the specific overexpression of α -AChR leading after several weeks of Poly(I:C) injections to an anti-AChR autoimmune response characterized by the production of anti-AChR antibodies, a specific proliferation of B cells and MG-like clinical signs. However, in this MG mouse model we do not observe the development of thymic GCs. In fact, thymic changes are transiently observed after 1 week of injections and disappear thereafter (18, 19).

The aim of the study was to find a combination of triggers to better recapitulate the human MG disease associated with thymic pathology including GC development. Compared to

other TLR agonists, such as those activating TLR4, TLR7 and TLR9, Poly(I:C) is not well known to favor the development of GCs as fully reviewed in **Table 1**. We then hypothesized that the combined activation of Poly(I:C) signaling pathway in the classical EAMG model or the combined use of Poly(I:C) with other TLR agonists could sustain thymic changes and favor the effective development of thymic GCs as observed in MG patients.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) unless indicated otherwise.

Animals

C57BL/6 female mice were purchased from Janvier Labs (Saint-Berthevin, France) and housed 1–2 weeks before experiments in a SPF animal care facility (CEF – Pierre and Marie Curie University, Paris, France). The study was approved by the local Ethics Committee (agreement no 2569.01).

TABLE 1 | Implication of toll-like receptor (TLR) signaling pathways in germinal center (GC) development in mice.

TLR implication in GC development in mice	
TLR1	<ul style="list-style-type: none"> TLR1 is expressed mainly on follicular-B cells compared to other naïve-B cells (36).
TLR3	<ul style="list-style-type: none"> TLR3 ligand enhances GC formation in the spleen during the tetanus toxoid vaccine response (35).
TLR4	<ul style="list-style-type: none"> TLR4 is expressed on follicular-B cells and other naïve-B cells (36). TLR4-mediated activation of B cells may help to feed and stabilize spontaneous and ectopic GCs (57). TLR4 signaling by follicular dendritic cells is central for GC onset (58).
TLR7	<ul style="list-style-type: none"> TLR7 is expressed on follicular-B cells and other naïve-B cells (36). TLR7 exerts B-cell intrinsic effects in promoting spontaneous GC and plasmablast B-cell development (39). Using TLR7 knockout mice, an essential role of TLR7 in spontaneous GC development is observed (40). TLR7 is essential for spontaneous GC response including B-cell expansion and diversification in lupus-prone mice (41). GC B cells in TLR7-deficient mice proliferated to a lesser extent (42). Extra copy of TLR7 (on the Yaa locus) relaxes the stringency for selection in the GCs resulting in increased autoreactivity of the antigen-driven B-cell repertoire (43). TLR7 ligand within virus-like particles largely restores defective GC reaction and antibody responses in IL-21R-deficient mice (44). TLR7 activation in follicular dendritic cells promotes autoreactive B-cell responses (45).
TLR9	<ul style="list-style-type: none"> TLR9 is expressed on follicular-B cells and other naïve-B cells (36). CpG is one of the most potent B-cell mitogens known (46). CpG DNA markedly augments plasma-B cell generation from GC-B cells (47). TLR9 signaling in dendritic cells and B cells controls the magnitude and quality of the GC response, respectively (48).

TLR Agonist Injections in Mice

6- to 8-week-old C57BL/6 mice were injected (i.p.) with Poly(I:C) 200 µg (tlrl-pic-5, Invivogen, Toulouse, France), Imiquimod 20 µg (tlrl-imqs, Invivogen), CpG (class C) oligodeoxynucleotides 25 µg (ODN 2395, Invivogen), lipopolysaccharide (LPS) 10 µg (ALX-581-007, Invivogen: low doses of LPS were used to avoid endotoxemia), or physiological water. For short-term experiments, mice were injected three times every other day and sacrificed the day after the last injection. For long-term experiments, mice were injected with TLR agonists every three days throughout the experiment.

Torpedo californica AChR (T-AChR) Extraction and Purification

250 g of frozen electric organ tissue from *T. Californica* (EastCoast Bio, North Berwick, ME, USA) were minced and homogenized at 4°C in few milliliters of buffer A [0.01 M Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM sodium azide (NaN₃), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 M NaCl]. The filtrate was centrifuged at 8,000 rpm for 30 min at 4°C. The pellet was resuspended in 400 mL of buffer B (0.01 M Tris, 1 mM EDTA, 0.5 mM NaN₃, and 0.1 mM PMSF) and centrifuged as above. The pellet was resuspended in a total of 8 mL of Buffer B supplemented with Triton X-100 and NaCl to a final concentration of 1% and 0.1 M, respectively. After stirring for 1 h at room temperature the suspension was centrifuged at 38,000 rpm for 1 h at 4°C. The supernatant was mixed with alpha-cobratoxin (L8114-1MG, Latoxan, Portes les valence, France) coupled to sepharose beads (as described below) and gently stirred overnight at 4°C for adsorption. The mix was filtered on a sinter glass once with 100 mL of Buffer A containing 0.1% Triton X-100, once with Buffer B containing 1 M NaCl and finally again with Buffer A containing 0.1% Triton X-100. The elution was performed by stirring the beads for 1–2 h with 10 mL of elution buffer made of 0.7 M carbamyl choline chloride (C-4382) in dialysis buffer. Eluent was then filtered on a sinter glass as described above. The purified receptor was dialyzed for 15 h at 4°C in 1–2 L of dialysis buffer (0.01 M Tris, 1 mM EDTA, 0.5 mM NaN₃, 0.1% Triton X-100, and 0.1 M NaCl). The receptor quantification was then performed by BCA assay and stored at –80°C until used.

Alpha-cobratoxin coupled to sepharose beads were prepared as followed. Sepharose beads (4 g) were mixed 15 min at room temperature with 40–50 mL of 1 mM HCl and filtered on a sinter glass with 100 mL of 1 mM HCl. Beads were washed with coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl), added to 15 mg of alpha-cobratoxin poulder dissolved in coupling buffer, and incubated for 2 h at room temperature. After filtration, the remaining active groups were blocked by adding about 100 mL of 0.2 M glycine and stirred 30 min at room temperature. The beads were washed with buffer A before used.

Experimental Autoimmune Myasthenia Gravis (EAMG)

Experimental autoimmune MG model was done mainly as described by Tuzun et al. with slight modifications (27). 6–8

week-old C57BL/6 mice were immunized with T-AChR. T-AChR was emulsified with an equal volume of CFA (F5881) supplemented with *Mycobacterium tuberculosis* (MTB) 10 mg/mL (H37RA, BD Difco, Villepinte, France). Mice were subcutaneously injected (200 μ L/mouse, 30 μ g AChR) at several sites (hind foot-pads, tail base, and in the back). After 4 weeks, mice were immunized a second time with T-AChR and CFA emulsion. A third immunization was done if the percentage of sick mice was less than 50%. Mice were euthanized 2–3 weeks after the last immunization for assessment of immunopathological parameters. Control mice were injected similarly with CFA emulsion devoid of T-AChR. Mice were regularly monitored for signs of muscle weakness, and mice that were too weak as defined by the ethical committee were euthanized.

Clinical Evaluation of Mice

Different assessments were taken into account to evaluate the clinical state of the animals. Mice were weighed every week. Muscle strength was analyzed by measuring the forelimb strength with a grip strength apparatus. As clinical signs are not always obvious in resting mice, the grip test measurements were done after a 3-min run on a treadmill. In experiments on the EAMG models, an inverted grid test was also carried out. Mice were tired by gently dragging them 20 times across the top grid of a cage and then carefully observed as the grid was rotated. The time at which the mouse falls-off, the behavior of mice is noted. Individually, these tests are not always powerful enough to determine if a mouse was sick. However, the combination of the different tests allows establishment of a global clinical score that is much more relevant. A global clinical score was then calculated as previously described taking into account the loss of weight, the grip test, and the grid test results for T-AChR-immunized mice compared to control mice. A mouse was considered sick when it reached a global clinical score of 2 (29). In long-term experiments using only TLR agonist injections, the clinical evaluations were done regularly. For the global clinical score evaluation, a rotarod test was done instead of the inverted grid test and a mouse was considered sick with a global clinical score above 3. In the course of the experiments, mice that were considered too sick were euthanized and classified with the higher global clinical score.

ELISA

To detect anti-AChR antibodies, 96-well ELISA plates were coated overnight at 4°C with 0.5 μ g/mL of T-AChR diluted in 10 mM NaHCO₃ buffer, pH 9.6. T-AChR-coated wells were blocked with 10% SVF in PBS at 37°C for 2–3 h. 100 μ L of mouse serum (1:100,000 for EAMG experiments or 1/100 for other experiments) or 5 μ g of thymic extracts were used per well and incubated for 90 min at 37°C. Subsequently, wells were washed four times with a PBS-0.05% Tween buffer. 100 μ L of 1/10,000 diluted biotinylated polyclonal anti-mouse IgGs (E0413, Dako, Courtaboeuf, France) were added for 90 min at 37°C. Next, samples were incubated with 100 μ L of streptavidin–horseradish peroxidase 1:10,000 (PN IM0309, Beckman Coulter, Villepinte, France).

To measure the expression level of all IgG, 96-well plates were coated overnight at 4°C with a polyclonal goat anti-mouse IgGs

(1 μ g/mL) (Z0420, Dako). After blocking for 1 h with PBS-BSA 2%, mouse serum (1:100,000) or standards (Clone DAK-GO1, X0931, Dako) were incubated for 90 min at 37°C and, subsequently, 1:10,000 diluted biotinylated anti-mouse IgGs and 1:10,000 streptavidin–horseradish peroxidase were added.

Tetramethylbenzidine was used for color development, and the optical density at 450 nm was measured with a microtiter plate reader spectrophotometer. The levels of anti-AChR antibodies were normalized on the total level of IgG.

Flow Cytometry

Single cell suspensions from thymus were prepared by passing the organs through a nylon mesh. Isolated cells were incubated for 30 min on ice with antibodies from BD Bioscience, except when specified: anti-CD19 PE (553786, clone 1D3 BD) or CD19-eFluor450 (48-0193-82, clone eBio1D3, eBioscience, Paris, France), anti-CD4 Alexa700 (557956, clone RM4-5 BD) or anti-CD4-APC (553051, clone RM4-5 BD), anti-CD8a PE-Cy7 (552877, clone 53-6.7 BD). Flow cytometry was performed on a FACS Verse (BD Biosciences) and data analyzed using FlowJo software.

Quantitative RT-PCR

Total RNA was extracted in TRIzol (Invitrogen) using the FastPrep FP120 instrument (Qbiogen, Illkirch, France). RNA (1 μ g) was reverse-transcribed for 1 h at 42°C using AMV (Ref 10109118001, Roche Life Science, Meylan, France) with oligo-dT (Invitrogen). RT-PCR reactions were performed with the LightCycler[®] 480 System (Roche). The primer sequences (Eurogentec, Angers, France) are listed in Table S1 in Supplementary Material. All samples were normalized to GAPDH.

Immunohistochemistry

Cryosections of thymic samples (7 μ m) were fixed in ice-cold acetone for 20 min and unspecific binding sites blocked with 2% BSA. Sections were stained with anti-K5-FITC polyclonal antibody (PRB-160P, Eurogentec) for medullary TECs, while B cells were detected with a biotinylated anti-B220 antibody (clone RA3-6B2, BD bioscience) and streptavidin Alexa-Fluor-594 (S11227, Invitrogen). Images were acquired with a ZeissAxio Observer Z1 Inverted Microscope. The number of B cells was counted in 6–8 fields representative of thymic sections.

Statistical Analyses

For 2-by-2 comparisons, non-parametric Mann–Whitney test was applied as specified in figure legends.

RESULTS

Effects of Poly(I:C) Injections on the Classical EAMG Model

In order to obtain a EAMG model associated with tertiary lymphoid genesis, we investigated the combined effects of Poly(I:C) injections together with the classical immunization protocol used to induce EAMG.

Two independent experiments were carried out and representative results are given in **Figure 1**. Mice immunized with T-AChR according to the classical EAMG protocol (CFA/T-AChR), displayed weight loss (**Figure 1A**), a decreased grip strength (**Figure 1B**) and taking into account the global clinical score, 50% of the animals were considered sick at the end of the experiment (**Figures 1C,D**). It is important to note that mice are quite resistant to the EAMG model and not all treated animals

get sick (27). Mice immunized with T-AChR in the presence of Poly(I:C) [CFA/TACHR/Poly(I:C)] displayed a worsening of clinical signs (**Figures 1C,D**). Using the global clinical score, we detected that seven out of eight animals (87.5%) were already sick after the first boost (**Figures 1C,D**; Figure S1A in Supplementary Material).

Although animals were clearly more affected when Poly(I:C) was injected, the levels of circulating anti-AChR measured were

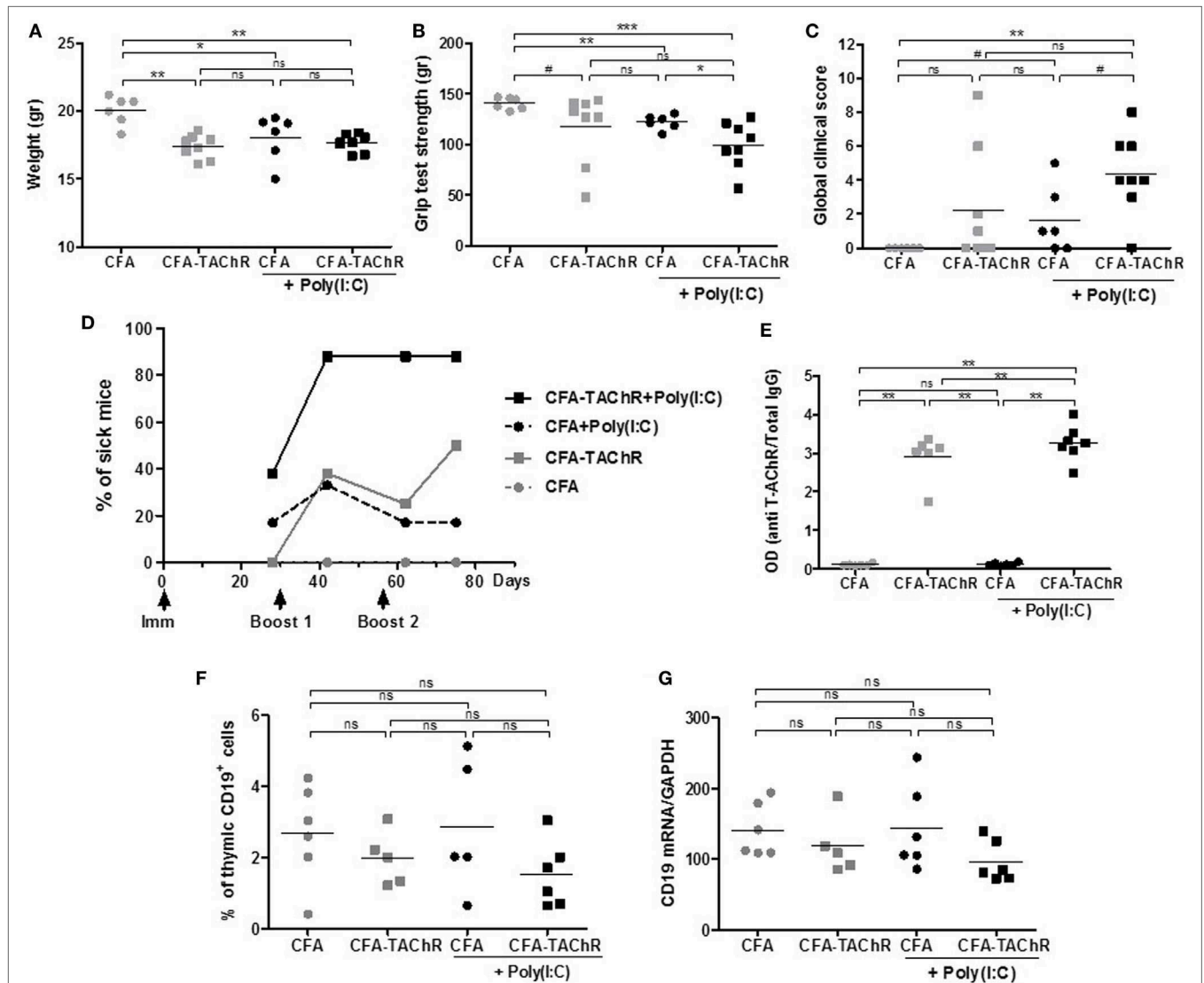


FIGURE 1 | Effects of polyinosinic-polycytidylic acid [Poly(I:C)] injections on the classical experimental autoimmune myasthenia gravis (EAMG) model. C57BL6 mice were immunized at day 0 and boosted twice at days 30 and 56 with complete Freund's adjuvant (CFA) or CFA/*Torpedo californica* AChR (T-AChR). i.p. injections of Poly(I:C) were done twice a week for the groups CFA/Poly(I:C) and CFA/T-AChR/Poly(I:C). Clinical evaluations were done at days 28, 42, 62, and 75, and data obtained at day 42 are shown on graphs A–C. **(A)** Mice were weighted. **(B)** Muscle strength was measured with the grip test apparatus after exercise on a treadmill. **(C)** A global clinical score for each mouse was calculated taking into account the weight loss, the grip test, and the inverted grid test. **(D)** The percentages of mice with a global clinical score of at least 2 and clear muscle weakness symptoms are shown in kinetic and statistical analyses were done to compare T-AChR groups to their respective CFA control groups. **(E)** ELISA for anti-AChR antibodies in serum taken 2 weeks after the last immunization. Data were normalized on the total level of IgGs. **(F)** Thymuses were analyzed by flow cytometry for the percentage of B cells (CD19⁺ cells) or **(G)** by PCR for the level of CD19 mRNA expression. *p*-Values were assessed by the Mann–Whitney test and annotated as follow on graphs: #<0.1, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

not really increased compared to animals immunized only with CFA/T-AChR (**Figure 1E**; Figure S1B in Supplementary Material). In the EAMG model, mice develop first large amounts of antibodies against the T-AChR and afterward autoantibodies against the mouse AChR that are probably more pathogenic (30). In the EAMG model, our ELISA test did not discriminate between mouse AChR and T-AChR autoantibodies but higher level of pathogenic mouse AChR antibodies upon Poly(I:C) injections could explain the increased MG symptoms.

In the control group corresponding to mice injected with CFA/Poly(I:C), we observed an induction of MG clinical signs with a comparable decrease in weight and grip strength and increase in the global clinical score compared to CFA/T-AChR mice (**Figures 1A–D**). As previously observed, Poly(I:C) is able to induce by itself the production of mouse AChR autoantibodies that can be detected by radioimmunoassay (18) and with our ELISA tests (data not shown). However, the levels of anti-mouse AChR antibodies induced by Poly(I:C) were 100–200 less than those produced by CFA/T-AChR injections and not detectable in the present ELISA tests in which serum was diluted at 1:100,000 (**Figure 1E**).

As Poly(I:C) worsened the MG symptoms in EAMG model, we analyzed the thymus of the animals at the end of the experiments. By flow cytometry, we did not detect more thymic B cells in the different subgroups (**Figure 1F**). By PCR, we did not observe either an increase in CD19 expression (**Figure 1G**).

Altogether these results demonstrated that Poly(I:C) injections greatly favor MG symptoms in the classical EAMG model as almost all animals were already sick after the first boost. However, we did not observe thymic changes at the end of the experiments suggesting that specific signal(s) was (were) missing to induce ectopic GC development as in the human MG disease. As Poly(I:C) is not considered to be involved in GC development, we investigated the effects of other TLR agonists (**Table 1**).

Effects of Imiquimod and CpG Together with Poly(I:C) Injections

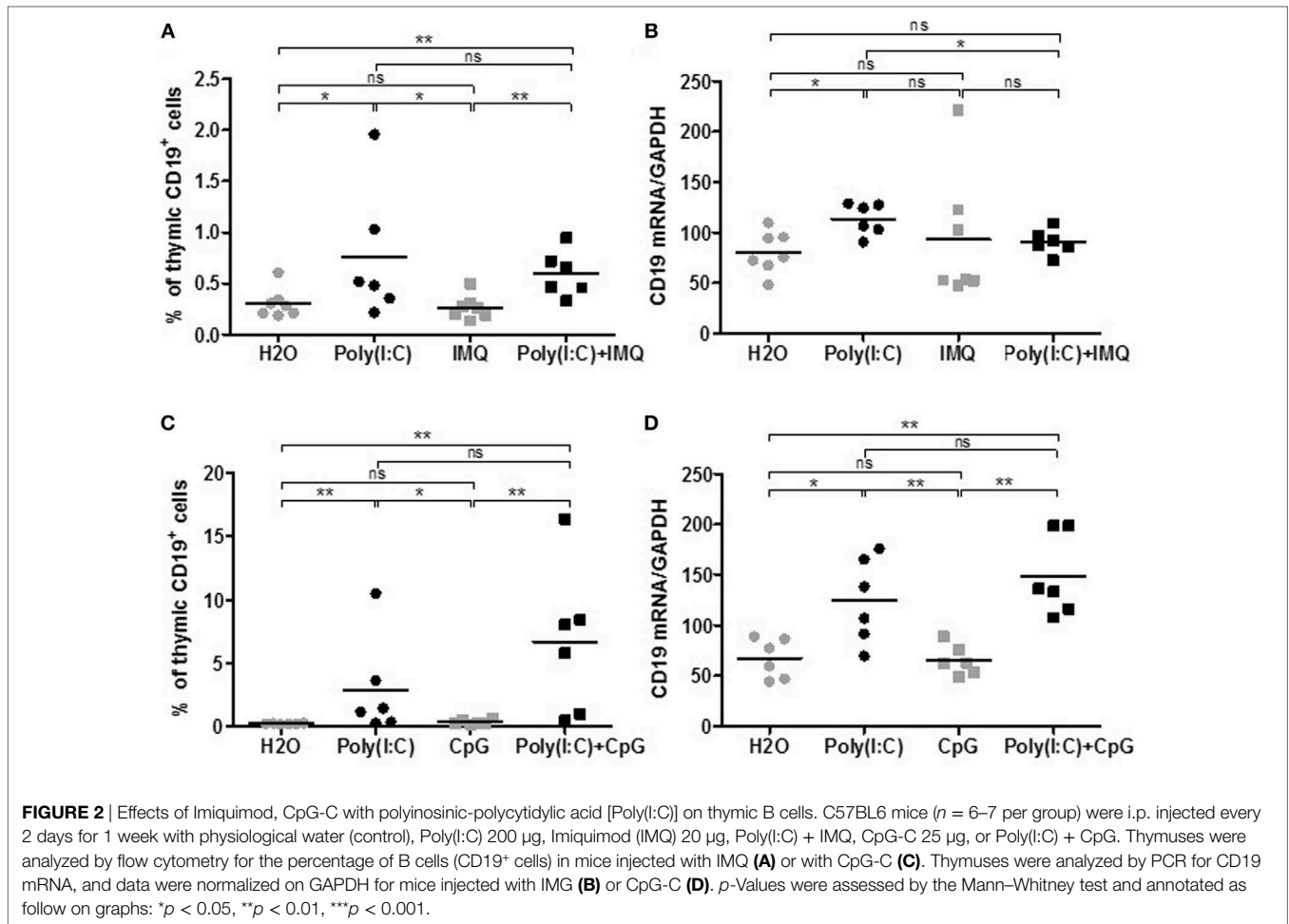
Polyinosinic-polycytidylic acid injections in mice are able to induce thymic changes after 1 week and MG symptoms associated with anti-AChR production after several weeks. However, we do not observe persistent thymic changes (18). We then investigated if we could exacerbate or sustain Poly(I:C) effects with simultaneous injections of agonists of other TLR, such as TLR7 and TLR9, that could be involved in GC formation (**Table 1**). TLR7 is activated by ssRNAs from viral infections. We used Imiquimod (**Figures 2A,B**), an imidazoquinoline amine analog to guanosine that specifically activates TLR7 but not TLR8. For TLR9 activation (**Figures 2C,D**), we used CpG oligodeoxynucleotides that correspond to short synthetic single-stranded DNA molecules containing unmethylated CpG dinucleotides mimicking bacterial DNA. We selected CpG class C that combines features of CpG class A favoring IFN- α production from plasmacytoid dendritic cells, and CpG class B inducing B-cell proliferation.

After 1 week of injections, analyzing the percentage of thymic B cells by flow cytometry (**Figures 2A–C**) or the expression by PCR of the B-cell marker CD19 (**Figures 2B–D**), we did not observe any effects of Imiquimod or CpG-C by themselves on the recruitment of thymic B cells. We did not either observe any additional effects when used with Poly(I:C). The same observations were made on CXCL13, CCL21, IFN- β , or α -AChR mRNA expression (data not shown). Consequently, we did not investigate further the effect of Imiquimod and CpG-C.

Effects of LPS Together with Poly(I:C) Injections

We next investigated if we could exacerbate Poly(I:C) with simultaneous LPS injections. Systemic administration of LPS is commonly used to study inflammation-associated behavioral changes in rodents. Moreover, LPS could also favor GC development (**Table 1**). After 1 week of injections, Poly(I:C) was able to increase the percentage of thymic B cells (**Figures 3A,B**) and thymic expression of CXCL13, CCL21, IFN- β , and α -AChR (**Figures 3C–G**), as previously demonstrated (18). We observed an increase in B cells in the thymus of LPS-treated mice by analyzing CD19 mRNA expression but not by flow cytometry (**Figures 3A,B**). We did not detect any effects of LPS by itself on the expression of CXCL13, CCL21, IFN- β , IFN- α , or α -AChR mRNAs in the thymus (**Figures 3C–G**). However, LPS in combination with Poly(I:C) induced a huge increase in B cells in the thymus of mice as observed by flow cytometry and by PCR analyses (**Figures 3A,B**). The thymic recruitment of B cells in LPS/Poly(I:C)-injected mice was accompanied by a high overexpression of CXCL13 mRNA (**Figure 3C**) but not especially with changes in CCL21, IFN- β , or IFN- α mRNA expression compared to Poly(I:C)-injected mice (**Figures 3D–G**). We did not observe either a combined effect of Poly(I:C) and LPS on α -AChR mRNA expression (**Figure 3F**).

These effects indicated that LPS could exacerbate Poly(I:C) effects on thymic CXCL13 mRNA expression and favor a higher recruitment of B cells into the thymus after 1 week of injection. We thus investigated the effects of prolonged injections of LPS and Poly(I:C) for 6 weeks on MG symptoms appearance and thymic changes. In contrast to Poly(I:C) which decreased the animal weight, LPS had no effect (**Figure 4A**). Poly(I:C) and LPS separately were both able to decrease the mouse strength, but no exacerbated effect was observed when Poly(I:C) and LPS were injected together (**Figure 4B**). Using the global clinical test, we observed that symptoms were induced by Poly(I:C) and not really affected by LPS (**Figures 4C,D**). Analyzing circulating anti-AChR antibodies, we measured that Poly(I:C) induced anti-AChR production of around two times compared to controls. The effects of LPS were much pronounced with anti-AChR antibody productions around 6–7.5 times without or with Poly(I:C), respectively (**Figure 4E**). It is known that LPS can induce a global increase in IgG but here even once normalized on total IgGs, we observed that LPS induced a preferential production of anti-AChR antibodies. However, we did not observe associated MG symptoms when LPS was used on its own. Most likely, LPS



induced a strong expression of anti T-AChR IgG1 antibodies that do not bind to complement and were not as pathogenic as IgG2 antibodies.

We then analyzed if thymic changes were observed in mice at the end of the experiments. No significant differences were observed on thymic weights (data not shown). However, we measured an increase in expression of CD19 mRNA (**Figure 5A**), CD20 mRNA (data not shown), and CXCL13 mRNA (**Figure 5B**) in the thymus of mice injected with LPS or Poly(I:C)/LPS. In parallel, by immunohistochemistry, we observed that LPS but especially Poly(I:C)/LPS injections induced a significantly higher recruitment of B cells to the thymus as determined by cell counting on thymic sections (**Figures 5C,E-H**), and the presence of B-cell clusters was also observed in the thymus of a few mice as shown in **Figure 5H**. In parallel, analyzing the levels of anti-AChR antibodies in thymic fragments (**Figure 5D**), we observed significant increases in anti-AChR antibodies in all treated groups, as in the serum (**Figure 4E**). Even if within the group of Poly(I:C)/LPS treated mice, some of them displayed higher levels of anti-AChR antibodies, they did not especially correspond to thymic fragments for which lymphoid aggregates were observed by immunohistochemistry. This last observation could be due to the fact that proteins were extracted from different fragments.

Altogether, these data suggest that thymic inflammation subsequent to LPS and Poly(I:C) signalization are important for tertiary lymphoid genesis in the thymus of mice.

DISCUSSION

As fully described in introduction, the MG thymus displays all the characteristics of TLOs. How the thymus acquired these features is not well-known but local inflammation seems to be mandatory. The overexpression in the MG thymus of IFN- β and IFN-I-induced genes suggests that MG could be triggered by pathogen infections. Inappropriate TLR signaling has been associated with various autoimmune diseases and more specifically persistent viral infection related to TLO development (18, 31, 32). Moreover, as detailed in **Table 1**, the activation of some TLR signaling pathway seems to play a central role in GC reactions. The overexpression of various TLRs has been observed in the MG thymus (24–26). Moreover, Poly(I:C) that activates TLR3 (but also other dsRNA-sensitive proteins) can induce in mice transient thymic changes recapitulating some characteristics of the MG thymus and lead to MG symptoms (18). Altogether, these data suggest that inappropriate TLR signaling activation might be responsible for ectopic GC development.

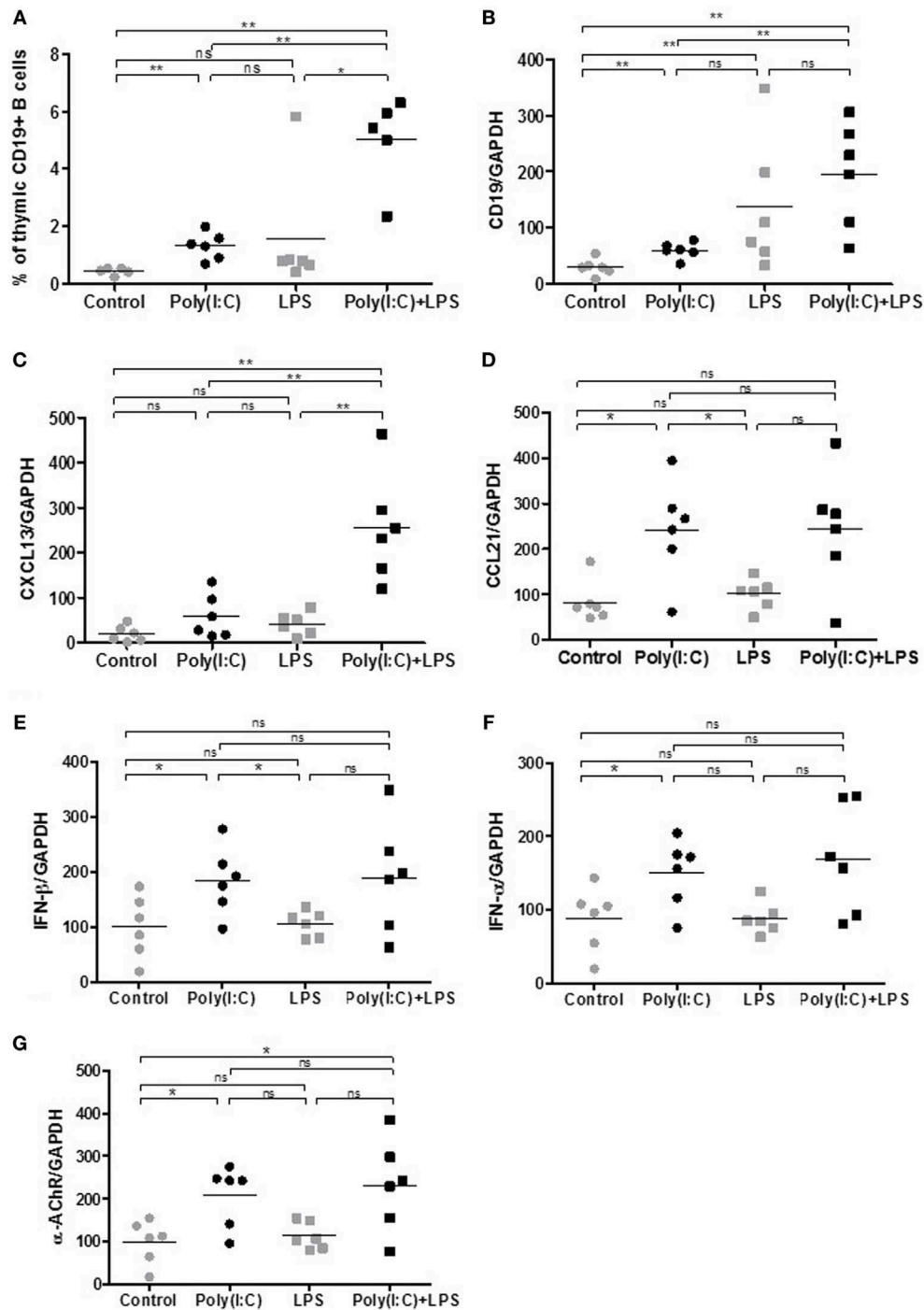
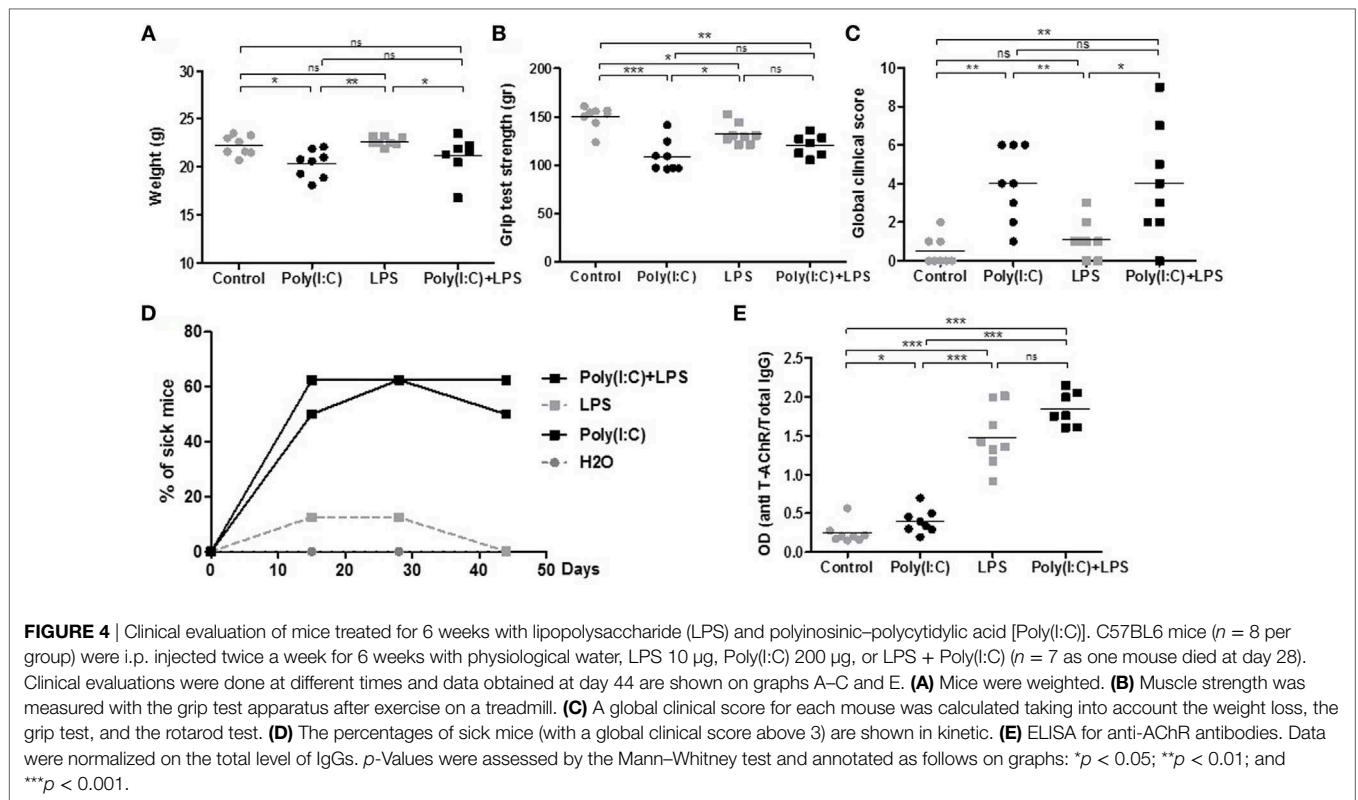


FIGURE 3 | Thymic changes in mice treated for 1 week with lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid [Poly(I:C)]. C57BL6 mice ($n = 6$ per group) were i.p. injected every 2 days for 1 week with physiological water, LPS 10 μg , Poly(I:C) 200 μg , or LPS + Poly(I:C). Thymuses were analyzed (A) by flow cytometry for the percentage of B cells (CD19⁺ cells) or by PCR for CD19 (B), CXCL13 (C), CCL21 (D), interferon (IFN)- β (E), IFN- α (F), and α -AChR (G) mRNA expression. Data were normalized on GAPDH. p -Values were assessed by the Mann-Whitney test and annotated as follows on graphs: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Attempt to Induce Thymic Lymphoid Neogenesis in the Classical EAMG Model

In the classical EAMG model induced by immunizing mice with T-AChR, mice are quite resistant to develop MG symptoms

despite the strong immune reaction triggered by the injections of T-AChR emulsified in CFA (27). In this model, mice develop antibodies against AChR associated with muscle weakness but this model does not recapitulate the human disease, as no



tertiary lymphoid neogenesis is observed (28). Our objective was to develop a new EAMG model associated with thymic tertiary lymphoid neogenesis. The basic principle of experimental models for autoimmune diseases involves a strong inflammatory reaction associated with the use of CFA. CFA contains heat-inactivated MTB that could activate TLR2, TLR4, TLR9, and possibly TLR8 signaling pathways (33). However, it appears not sufficient to lead to thymic inflammation and GC development in the EAMG model.

As i.p. injections of Poly(I:C) can induce transient thymic changes (18), we challenged the EAMG model with concomitant Poly(I:C) injections. We observed that Poly(I:C) favored MG development as almost all mice had an elevated clinical score associated with MG symptoms. Unfortunately, even if Poly(I:C) exacerbated MG development in the EAMG model, we did not observe the induction of thymic changes associated with B-cell recruitment and ectopic GC development.

Lipopolysaccharide injections have also been used in the EAMG mouse model to try to substitute CFA as MTB can activate TLR4. 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. AChR-LPS/IFA-immunized mice develop clinical signs that are similar to those observed with the AChR/CFA immunization but B-cell activation leading to the production of the anti-AChR antibodies is different in AChR-LPS/IFA and AChR/CFA models. They demonstrated that CFA immunization requires CD4 costimulation of B cells to induce anti-AChR IgG2 secretion while LPS immunization does not. Unfortunately, in this study the ability

of LPS to induce GC development in the thymus has not been investigated (34).

Despite the strong inflammatory effects of CFA, transduced whether or not by TLRs, it appears not sufficient to lead to thymic inflammation and GC development in the EAMG model even in association with Poly(I:C). This might be a consequence of the administration mode. Indeed, the injection of antigen-protein in CFA induces a local inflammation that might not efficiently impact the thymus.

No Effects of TLR7 and TLR9 Agonists in Inducing or Sustaining Poly(I:C) Effects on Thymic B-Cell Recruitment

Regarding the early effects of Poly(I:C) on the mouse thymus and its late effects associated with anti-AChR antibodies production (18), our objective was to test the combined effects of Poly(I:C) with other TLR agonists: (1) to induce thymic tertiary neogenesis and (2) to develop a new MG mouse model that would be less stressful than the classical EAMG model using immunizations with CFA.

Only a few publications mentioned that TLR3 ligand enhances GC formation, as for example in the spleen during the tetanus toxoid vaccine response (35). In mice, TLR3 is weakly expressed on B-cell subsets compared to TLR1, TLR7, and TLR9, and it is especially detected on marginal zone B cells. Moreover, Poly(I:C) fails to stimulate B-cell proliferation (36). Consequently, Poly(I:C) might not be efficient enough to favor GC reactions. Except TLR3 that signals only through TRIF (TIR domain-containing

adapter-inducing IFN- β), all TLRs interact with the intracellular adaptor myeloid differentiation primary response gene 88 (MyD88) to activate downstream signaling cascades. In mice, TLR signaling *via* MyD88 in B cells is supposed to enhance antibody response and favor GC development (37). It is also necessary to maintain long-lived plasma cells (38). We then used combined i.p. injections of Poly(I:C) with TLR7, TLR9, and TLR4 agonists (Imiquimod, CpG-C, and LPS, respectively), knowing that these TLR receptors are abnormally expressed in the thymus of MG patients (18, 24–26).

Imiquimod did not modify Poly(I:C) effects on the mouse thymus. This observation was very surprising as TLR7 signaling activation seems to be crucial for spontaneous GC development and plasmablast B-cell development (39–43). TLR7 is expressed on different B-cell subsets (36) and could regulate key checkpoints controlling GC development (44). Das et al. also recently demonstrated that TLR7 on follicular dendritic cells promotes autoreactive B-cell responses (45).

We have used CpG-C allowing both the production of IFN- α by plasmacytoid dendritic cells and B-cell stimulation. TLR9 activation in combination with Poly(I:C) was not effective either in sustaining Poly(I:C) effects on the mouse thymus. This observation was again very surprising as CpG is known to directly activate B cells and is one of the most potent B-cell mitogens known (46). *In vitro*, CpG stimulates plasma cell differentiation of naïve and memory B cells and can synergize with B-cell receptor (BCR) cross-linking

to dramatically enhance B-cell proliferation (47). Rookhuizen and DeFranco have also demonstrated that TLR9 signaling acts in multiple complementary ways to increase the magnitude and enhance the quality of GC reactions (48). Moreover, a synergistic engagement of BCR and TLR9 signaling has been shown to effectively activate autoreactive B cells *in vitro* (49). However, TLR9 engagement could be more complex. In fact, the implication of TLR9 activation in mouse models has complex consequences as TLR9 on B cells restrains TLR7-mediated spontaneous autoimmunity in C57BL/6 mice (50). Moreover, repeated TLR9 activation has also been shown to disrupt GC formation (51).

High expression levels of TLR7 and TLR9 proteins have been observed in B cells and plasma cells of thymic GCs and lymphoid infiltrates in MG (25, 52). In the MG thymus, the expression of these TLRs colocalized with Epstein-Barr virus (EBV) antigens, and it has been suggested that EBV-associated TLR7 or TLR9 signaling may promote abnormal B-cell activation and proliferation leading to GC formations. As human is the only natural host of EBV infection, it can explain why we were not able to sustain Poly(I:C) effects on mouse thymus using TLR7 and TLR9 agonists.

Combined Use of LPS and Poly(I:C) Induced Thymic B-Cell Recruitment

TLR4 is also overexpressed in MG thymuses (23, 24). Using LPS together with Poly(I:C) was very effective in potentiating some of

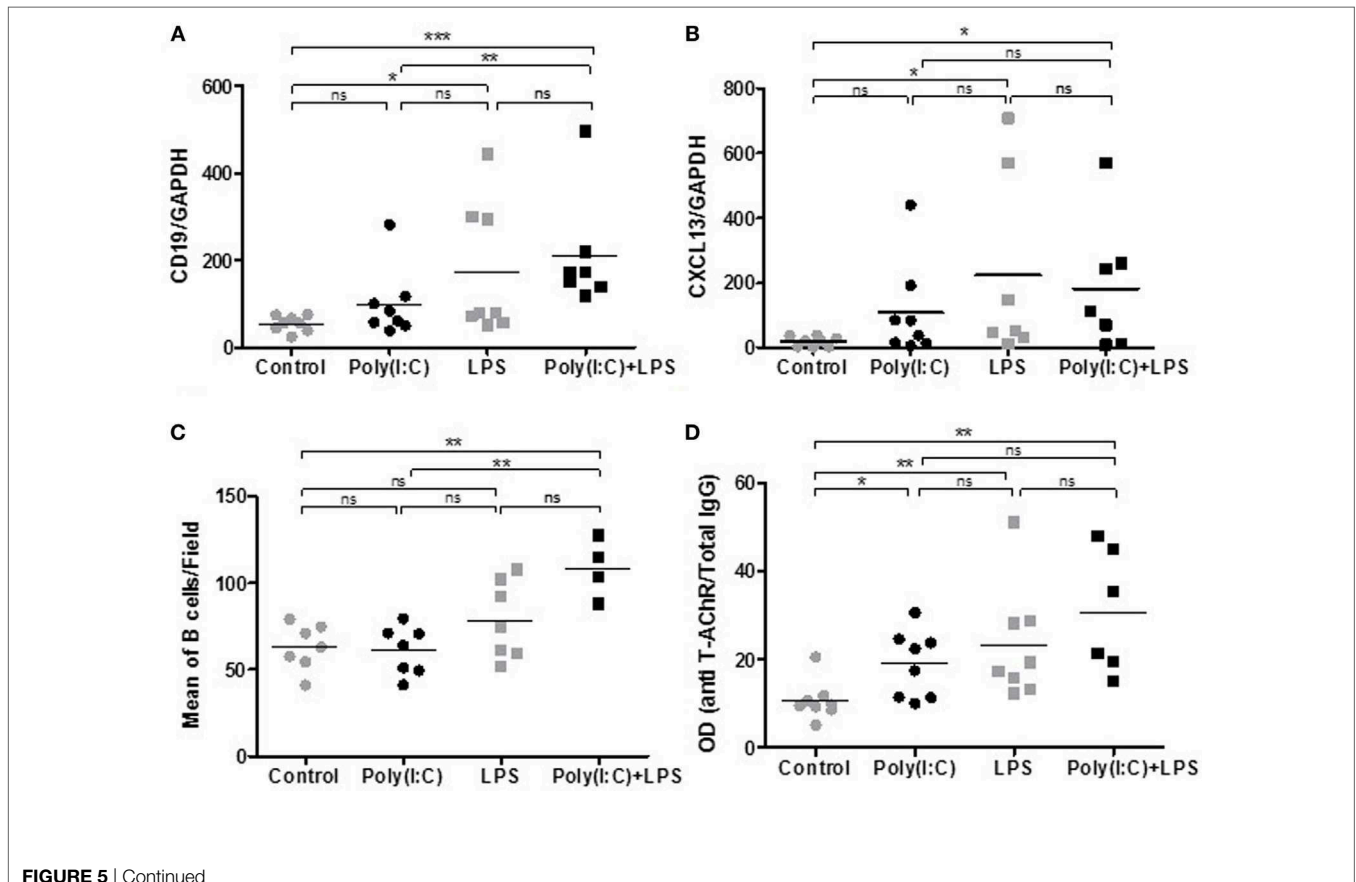


FIGURE 5 | Continued

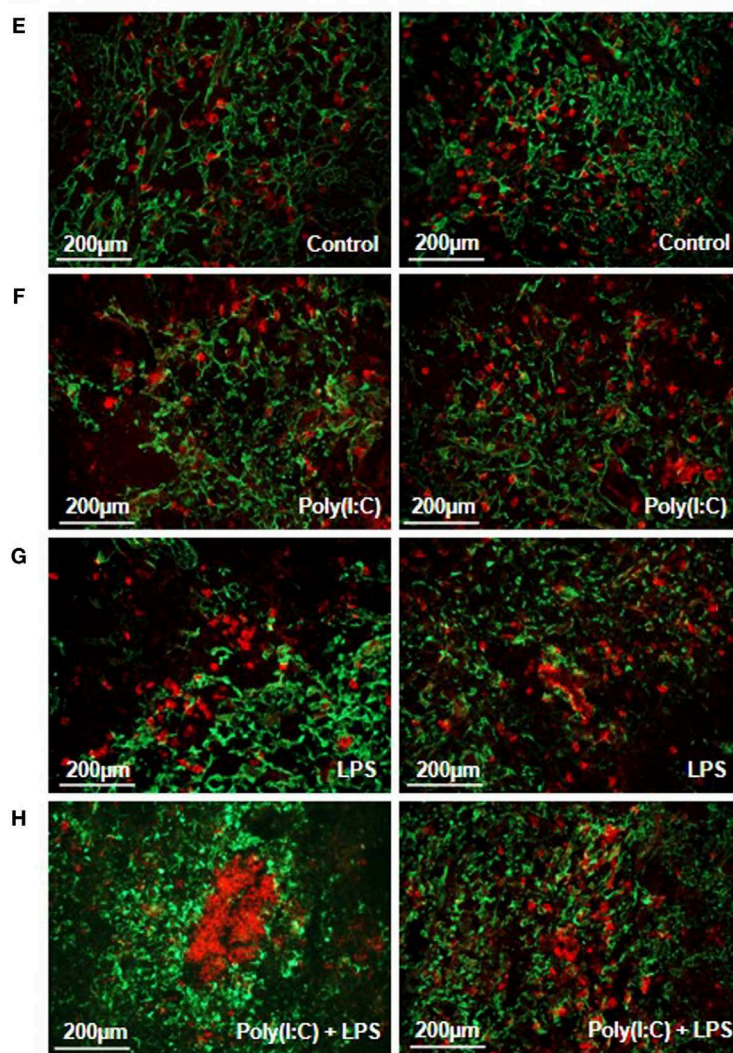


FIGURE 5 | Analyses of thymic changes in mice treated for 6 weeks with lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid [Poly(I:C)]. C57BL6 mice ($n = 8$ per group) were IP injected twice a week for 6 weeks with physiological water, LPS 10 μg , Poly (I:C) 200 μg , or LPS + Poly(I:C) ($n = 7$ as one mouse died at day 28). **(A,B)** PCR analyses for CD19 and CXCL13 mRNA. Data were normalized on GAPDH. **(D)** ELISA for anti-AChR antibodies on thymic extracts. Data were normalized on the total level of thymic IgGs. **(E–H)** Thymic sections were stained with an anti-K5-FITC antibody (green) and a biotinylated anti-B220 antibody plus a streptavidin Alexa-Fluor-594 (red). **(C)** The number of B cells was counted in 6–8 fields representative of thymic sections and each point correspond to the mean for each mice. In the Poly(I:C)/LPS group, two mice with B-cell clusters were excluded from the counting. **(E–H)** Representative thymic sections show in **(G)** the presence of B-cell clusters. p -Values were assessed by the Mann–Whitney test and annotated as follow on graphs: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.01$.

the Poly(I:C) effects. After 1 week of coinjections, we observed an increased thymic recruitment of peripheral B cells associated with a higher expression of the B-cell chemokine CXCL13. A higher expression of CXCL13 has also been observed in mice upon LPS-induced acute endotoxin stress (53). This potentiating effect seems independent of IFN-I signaling as LPS did not induce IFN- β or IFN- α expression. We next investigated the effects of prolonged injections of LPS and Poly(I:C). LPS alone or LPS with Poly(I:C) did not have much effects on the clinical evaluations compared to controls or Poly(I:C) alone, respectively. However, surprisingly, we observed an increased expression of anti-AChR antibodies due to LPS but this increased expression

did not induce more symptoms. In MG patients, there is no clear correlation between the anti-AChR titer and the severity of the disease, although such a correlation might be observed at the individual level.

Analyzing the thymic changes after prolonged coinjections of Poly(I:C) and LPS, we still observed a higher expression of CXCL13 mRNA associated with a higher proportion of thymic B cells and eventually the appearance of B-cell clusters in the thymus. In future experiments, markers should be used to investigate GC structure.

To further investigate lymphoid neogenesis in Poly(I:C)/LPS-stimulated mice, we also analyzed thymic sections for HEV

development but did not detect HEVs (data not shown). HEVs are well known to allow the entrance of lymphocytes within SLOs and TLOs (1). However, HEVs are not always mandatory for cell entrance in peripheral tissues. For example, in the spleen that exceptionally does not possess HEVs, lymphocytes directly pass from the blood to the red pulp and afterward migrate to the white pulp. In the hyperplastic thymus of MG patients, even if HEVs are largely found, we also demonstrated the presence of CCL21 positive lymphatic vessels that could serve as afferent lymphatic vessels and allow the recruitment of peripheral B and T cells (8). In a transgenic mouse model with the specific overexpression of CCL21 in the thyroid, Martin et al. observed the development of HEVs in the thyroid of transgenic mice but they demonstrated that HEVs were not required for initial colonization of the thyroid by lymphocytes and even suggested that HEV formation may depend on the infiltrating cells and could be a later event on the formation of the lymphoid aggregates (54).

How LPS can sustain Poly(I:C) effects increasing B-cell recruitment associated with lymphoid aggregates? LPS has been known for long to be a potent polyclonal activator for mouse B cells (55). Its receptor TLR4 is expressed on all mouse B-cell subsets. However, at lower levels compared to TLR1, TLR7, and TLR9 (36). TLR4 stimulation increases B-cell polarization, migration, and directionality, especially in response to CXCL13 (56). Hwang et al. nicely showed that TLR4 signaling enhances B-cell trafficking into lymph nodes, induces B-cell clustering and interactions within lymph node follicles, leads to sustained B-cell proliferation, overcomes the restriction that limits the access of non-antigen activated B cells to GC dark zones, and enhances the generation of memory and plasma cells (57). Moreover, Garin et al. demonstrated that TLR4 is expressed on follicular dendritic cells and TLR4 plays an important role in their maturation and the proper development of GC reactions (58).

Poly(I:C) through thymic expression of IFN- β is able to induce the expression of MG autoantigen, the α -AChR, but also the higher expression of CXCL13 and CCL21, known to favor GC development (18). Our data suggest that LPS through its effects on CXCL13 and B-cells sustained Poly(I:C) effects favoring the higher recruitment of B cells and the development of B-lymphoid aggregates.

We previously observed using transgenic mice with thymic overexpression of CXCL13 that CXCL13 itself is not able to induce thymic B-cell-related changes associated with MG. We then demonstrated that inflammation is mandatory to reveal CXCL13 ability to recruit B cells and to induce B-lymphoid structures (29). Here, even if CXCL13 was highly expressed and inflammation triggered by LPS/Poly(I:C) injections, B-lymphoid structures were only observed in a few mice, and no HEVs were observed by immunohistochemistry. Here, the same observation was done as we only observed a few lymphoid aggregates. It is important to mention that in human not all MG patients display GCs either (4, 59). Moreover, we cannot exclude the fact that the thymus might be more refractory to TLO development in mice compared to human. In addition, the genetic background involved in human MG or other autoimmune diseases is not recapitulated in rodents. We can also set up the hypothesis that other factors might be needed for GC development. Lymphotoxin alpha or

beta have been proposed to play a role but these molecules were not induced in the thymus of mice in our different experiments (data not shown).

CONCLUSION

The implication of TLR agonists in autoimmune experimental models has been largely studied for their adjuvant effect or their direct effect in inducing or exacerbating these models. However, the potential role of TLR agonists in the development of ectopic lymphoid structures in organ-specific autoimmune diseases has been less investigated or maybe not observed. For example, in Sjögren's syndrome, Nandula et al. demonstrated that repeated injections of Poly(I:C) to NZB/WF1 mice accelerate the development of salivary gland disease by causing a significant upregulation in chemokine expression and inflammatory cell infiltrations within the submandibular glands. However, the development of lymphoid structures was not described (60). In contrast, in MLR/Mp mice which spontaneously develop pancreatitis in the exocrine pancreatic tissues, Poly(I:C) accelerates the development of the disease and induce peripheral cell infiltration and lymphoid structure development in the pancreas (61). Regarding LPS, its direct implication in lymphoid neogenesis has also been punctually shown, as repeated intranasal administrations to neonatal C57BL/6 mice induces inducible bronchus-associated lymphoid tissue that displays TLO features (62).

Altogether these data demonstrated that tertiary lymphoid neogenesis is a complex process. In our experiments, even if thymic changes are induced by Poly(I:C) and sustained by LPS. Optimum GC reaction might be altogether species dependent, organ dependent, and/or TLR signaling pathway dependent. It has already been suggested that coactivation of extracellular and intracellular TLR by CpG and LPS may accelerate systemic autoimmune disease in the anti-dsDNA transgenic mice *in vivo* (63). Here, the efficacy of combined effects of Poly(I:C) and LPS also suggest that coinfection in individual could be at the base of particular inflammatory reactions leading to ectopic GC development associated with autoimmune diseases.

ETHICS STATEMENT

Experiments on mice were approved by the local Ethics Committee (agreement no. 2569.01).

AUTHOR CONTRIBUTIONS

MR, BV, SM, and MAC performed and analyzed the experiments, read and revised the manuscript. SB-A read and revised the manuscript. RLP conceptualized the study, analyzed the results, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.01029/full#supplementary-material>.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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)- β , 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- β

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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- α - or IFN- β -based therapies [32]; (2) antibodies against IFN- α 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- α 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- β , are overexpressed in the thymus of MG patients [38]. Analyzing in detail the *in vitro* effect of IFN- β , it is shown that IFN- β induces specifically the expression of α -AChR and not that of other TSAs. It also increases TEC death and the uptake of TEC proteins by DCs. In parallel, IFN- β 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- β 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- β and IFN- α 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- β and IFN- α 2 are overexpressed [39]. Altogether, these results demonstrate that IFN- β could play a central role in thymic events leading to MG by triggering the overexpression of α -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- α 2, IFN- α 8, IFN- ω , and to a lesser extent of IFN- β 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 α -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.

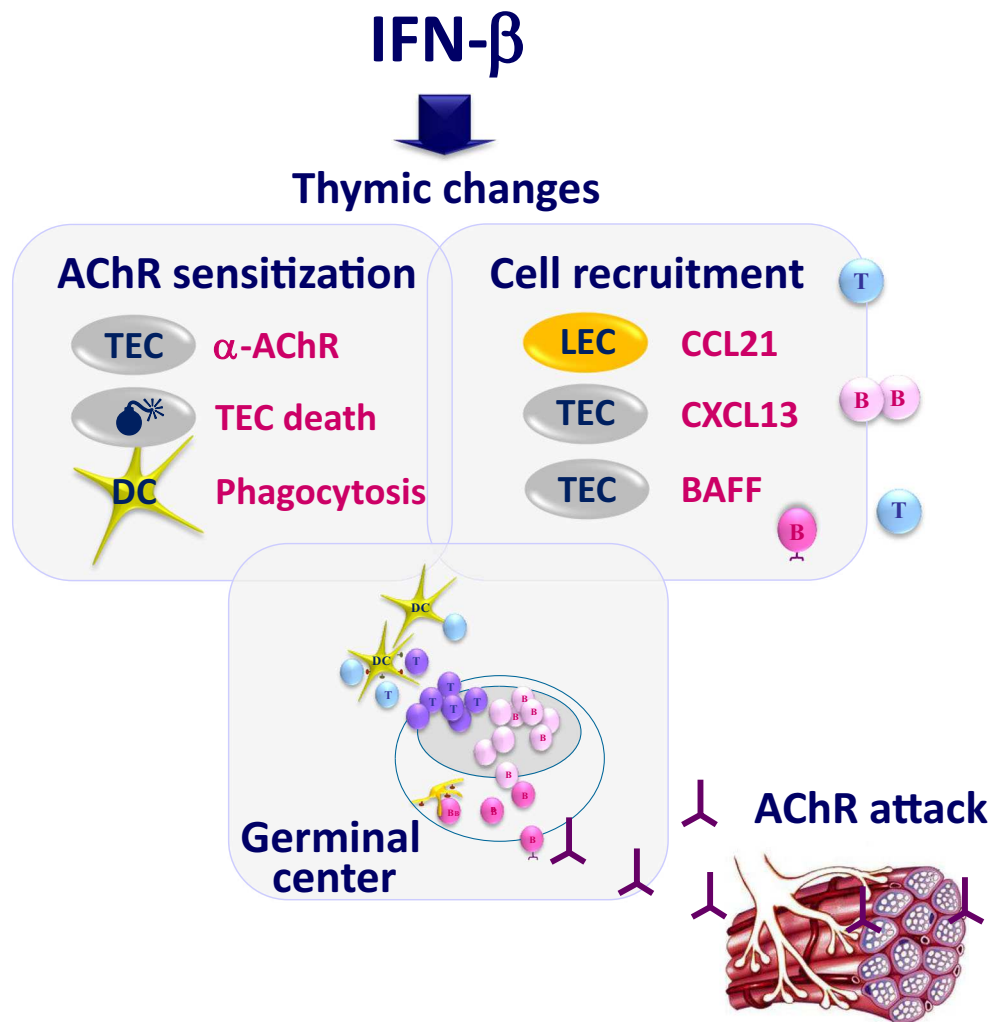


Fig. 1 Central role of IFN- β in thymic changes associated with MG. IFN- β induces the expression in TECs of α -AChR, the main autoantigen in MG. This effect is very specific, as IFN- β does not induce the expression of other AChR subunits or other tissue-specific antigens. IFN- β also induces TEC death and the uptake of TEC proteins by DCs, suggesting a potential sensitization of DCs to the α -AChR [39]. In parallel, IFN- β 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- β [39]. Altogether, these results demonstrate that IFN- β plays a central role in thymic events leading to MG by triggering the overexpression of α -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- β (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- κ 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- β 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- β [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 α -AChR but not other AChR subunits or TSAs. This induction is mediated through TLR3, PKR, and by the release of IFN- β but is completely inhibited by IFN-I receptor or IFN- β -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 μ 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- α , interleukin (IL)-12, IL-6, IFN- γ , 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⁺ T cells that secrete Th1 pro-inflammatory cytokines such as IFN- γ and the activation of CD8⁺ 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- γ 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⁺ 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].

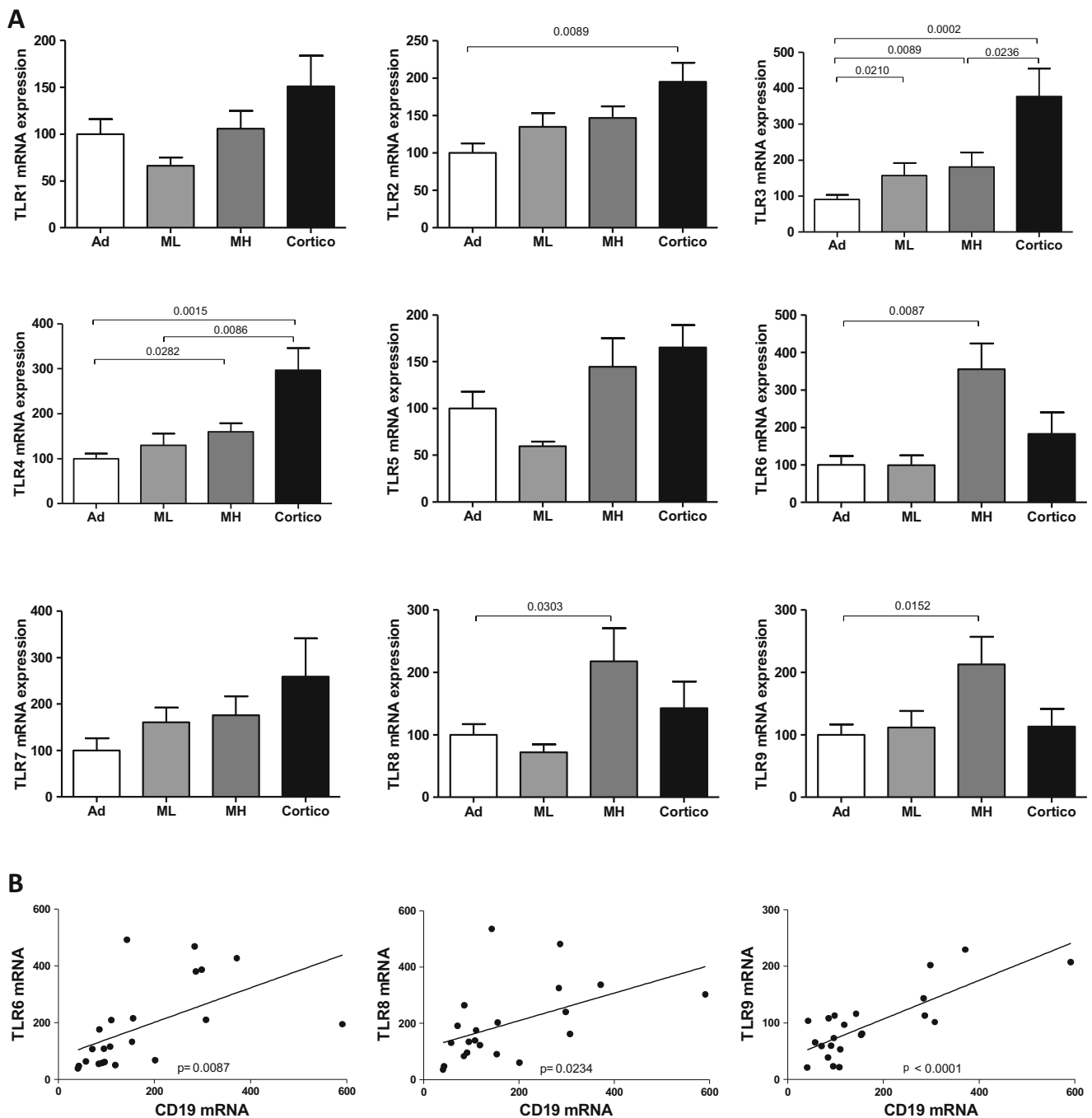
Allman et al. have used LPS as adjuvant instead of MTB in CFA to induce the EAMG model [101]. C57BL/6 mice were immunized by several subcutaneous injections in foot pads and shoulders with T-AChR and LPS emulsified in IFA or T-AChR emulsified in CFA. Mice were immunized three times at days 0, 28, and 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^{-/-} 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 Chirurgial 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 α -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- β . Interestingly, the overexpression of TLR3, PKR, IRF-7, IRF-5, and IFN- β , 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 μ 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- β ,

IFN- α 2, and α -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)	α -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)	β 2-glycoprotein I (β 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 α -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, α -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- β [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- β 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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