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Physiopathologie & Approches Thérapeutiques*

Study of Interferon type I in Myasthenia Gravis

Etude de l'interféron de type I dans la Myasthénie Autoimmune

Par Cloé PAYET

Thèse de doctorat de Biologie

Dirigée par le Dr. Rozen LE PANSE

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Abbreviations

AChR: Achetylcholine receptor

AGS: Aicardi–Goutieres syndrome

cGAS: cyclic GMP-AMP Synthase

DAI: DNA-dependent activator of IFN-regulatory factor

DAMP: Damage-associated molecular pattern

DDX41: DEAD-BOX Helicase 1

DNA-PK: DNA-dependant protein kinase

dsDNA: Double-strand DNA

dsRNA: Double-strand RNA

EAMG: Experimental autoimmune MG

EOMG: Early onset Myasthenia gravis

GC: Germinal center

HEV: High endothelial vessel

IC: Immune complex

IFI16: Gamma-interferon protein 16

IFN: Interferon

IFNAR: Interferon- α/β receptor

IL: Interleukin

IRF: IFN regulatory factor

ISG: Interferon stimulated gene

IVIg: Intravenous immunoglobulin

KIR: Kinase inhibitory region

LOMG: Late-onset Myasthenia Gravis

LPS: Lipopolysaccharide

LRP4: Lipoprotein related 4

MAVS: Mitochondrial antiviral-signaling protein

MDA5: Melanoma differentiation-associated protein 5

MG: Myasthenia Gravis

MGT: Thymoma-associated MG

MHC: Major histocompatibility complex

miRNA: MicroRNA

MS: Multiple sclerosis

MuSK: Muscle-specific kinase

Myd88: Myeloid differentiation primary response 88

NA: Nucleic acid

NK: Natural killer

NMJ: Neuromuscular junction

NSG: NOD scid gamma C KO

PAMP: Pathogen-associated molecular pattern

pDC: Plasmacytoid dendritic cell

PKR: Protein kinase R

PRR: Pattern recognition receptor

RA: Rheumatoid arthritis

RIG-1: Retinoic acid-inducible gene I

RLR: Rig-like receptor

RVCL: Retinal vasculopathy with cerebral leukodystrophy

SAVI: STING associated vasculopathy

SIMOA: Single-molecule array

SLO: Secondary lymphoid organ

SOCS: Suppressors of cytokine signaling

SPENCD: Spondyloenchondrodysplasia

ssRNA: Single-strand RNA

STAT: Signal Transducers and Activators of Transcription

STING: Stimulator of Interferon Gene

TBK1: TANK Binding Kinase 1

TCR: T cell antigen receptor

TEC: Thymic epithelial cell

Tfh: T follicular helper cell

Th17: T cell helper 17

TLR: Toll like receptor

Treg: Regulatory T cell

TRIF: TIR-domain-containing adapter-inducing interferon- β

TYK: Tyrosine kinase

TSA: tissue-specific antigen

USP18: Ubiquitin specific peptidase 18

Introduction

1. Interferon

1.1. Generalities

1.1.1. Interferon subgroups

In 1957, a soluble molecule able to regulate the resistance to viral infection was discovered (1957). This molecule is called interferon (IFN) because it interferes with viral replication. The IFN family is composed of three subgroups categorized according to their receptors: IFN type I, type II, and type III. These subgroups have all their own biological activities and specificities. IFN type I (IFN-I) is composed of 13 IFN- α isoforms, and only one isoform for IFN- β , IFN- ω , IFN- ϵ , and IFN- κ , and their genes are on chromosome 9 in human (Fountain et al., 1992). IFN-I subtypes have 30% to 85% of homologies within a species (Borden et al., 2007) and there are 70-80% of homologies in IFN- α isoforms. All mammalian species have the IFN- β gene and at least one IFN- α isoform. All cell types can produce IFN- α , IFN- β , and IFN- ω but IFN- ϵ and IFN- κ are tissue-specific (LaFleur et al., 2001) (Xi et al., 2012).

IFN type II is composed of only one isoform: IFN- γ (JaquelineDe Maeyer-Guignard, 1992). IFN subtype-III is the latest IFN family discovered (Kotenko et al., 2003) and is composed of IFN- λ 1 (also known as interleukin (IL)-29), IFN- λ 2 (IL-28a), IFN- λ 2 (IL-28b), and IFN- λ 4.

My PhD project is centered on IFN-I. The rest of my thesis manuscript will focus on this subgroup of IFN.

1.1.2. IFN-I-producing cells

In the case of pathogen infection, almost all the cells can produce IFN-I. However, plasmacytoid dendritic cells (pDC) are considered the primary source of IFN-I and are also called "IFN-I producing cells" (Cella et al., 1999). If pDC are the main sources of IFN-I for systemic infection, they are not very effective in local infection (Swiecki and Colonna, 2011). Other immune cells can express IFN-I upon infection, such as organ-specific macrophage subtypes like microglia cells. Neurons can become potent IFN-I producers if the brain is infected by a virus (Delhaye et al., 2006; Rosato and Leib, 2015).

For pulmonary infection, alveolar macrophages are the primary source of IFN-I (Kumagai et al., 2007). Some non-hematopoietic stromal cells, such as fibroblasts and epithelial cells, can also express IFN-I (Swiecki and Colonna, 2011).

1.2. Inducers of IFN-I expression

1.2.1. Pathogen and danger signals

To produce IFN-I, cells must detect a pathogen-associated molecular pattern (PAMP) associated with a pathogen (bacteria or virus), such as part of a bacterial wall, proteins, lipids, or nucleic acids (Akira et al., 2006). These PAMP are recognized by pattern recognition receptors (PRR) composed of Toll-like receptors (TLR), Rig-like receptors (RLR), NOD-like receptors (NLR), and intracellular DNA sensors. Injured or dying cells can release endogenous molecules called “damage-associated molecular patterns” (DAMP) that can also trigger IFN-I production. DAMP can be nuclear components, such as endogenous DNA or RNA, or cytosolic proteins, and are recognized by the same PRR as PAMP. It is called sterile inflammation because there is no infection (Chen and Nuñez, 2010).

1.2.1.1. Implication of nucleic acids in IFN-I expression

Double-strand RNA (dsRNA) was the first nucleic acid discovered to be implicated in the induction of IFN-I (1963). dsRNA can come from pathogens or correspond to self RNA deriving from damaged cells (Bernard et al., 2012). Endosomal dsRNA are recognized by TLR3 present in the endosomal membrane, whereas cytoplasmic dsRNA are recognized by protein kinase R (PKR) and two cytoplasmic RNA helicases, retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) (Yoneyama et al., 2004). RIG-1 and MDA5 are expressed at a low level in resting cells but are greatly upregulated in the case of a viral infection. To avoid sensing of self RNA, RIG-1 recognized preferentially RNA sequences with 5'triphosphorylated (Hornung et al., 2006), MDA5 recognized long dsRNA, not present in the cytoplasm of non-infected cells (Kato et al., 2008), and PKR recognized non-coding RNA and mitochondrial RNA (Kim et al., 2018). Single-strand RNA (ssRNA) is also a potent IFN-I inducer and is recognized by TLR7 and TLR8 in endosomes (Heil et al.,

2004). TLR7 showed an ability to recognize viral ssRNA in all expressing cells, and also bacterial ssRNA but only in conventional DC (Mancuso et al., 2009). TLR3, TLR7, and TLR8 have a low probability of encountering self-RNA molecules because of their endosomal location. However, TLR3 and TLR7 can also be located on the cell surface but with a lower expression compared to the endosomal location (Agier et al., 2017). TLR3 at the membrane plays a role in the recognition of extracellular dsRNA released from dying cells. Interestingly, in mice, activation of TLR3 at the cell surface does not activate IFN-I expression (Suresh et al., 2016).

The immunostimulatory and anti-viral potential of DNA has been known for a long time but the mechanism behind it was discovered only a few years ago. Both pathogenic and self-DNA can be immunostimulatory. Endosomal TLR9 was the first shown to be implicated in the recognition of CpG DNA, a bacterial DNA (Hemmi et al., 2000). In the last 20 years, recognition receptors other than TLRs have been discovered. A key component of DNA signalization is Stimulator of Interferon Genes (STING) (Ishikawa and Barber, 2008). It is not a DNA sensor by itself but part of the signaling cascade for different cytosolic DNA sensors: Gamma-interferon protein 16 (IFI16), DEAD-BOX Helicase 1 (DDX41), DNA-dependant protein kinase (DNA-PK), MRE11, DNA-dependent activator of IFN-regulatory factors (DAI), and the last one discovered, cyclic GMP-AMP Synthase (cGAS) (Dempsey and Bowie, 2015; Sun et al., 2013). If TLR can be cell-specific, such as in DC, macrophages, or B cells, non-TLR DNA cytosolic sensors are ubiquitously expressed (Wu and Chen, 2014).

1.2.1.2. Other pathogen molecular motifs inducing IFN-I

IFN-I is induced not only by nucleic acids. Lipopolysaccharide (LPS), a component of bacteria Gram-negative membrane, is a potent inducer of IFN- β in macrophages and DC (Jacobs and Ignarro, 2001). LPS is recognized by TLR4, found at the membrane surface of the cell. It was also shown that iE-DAP, a dipeptide present in the peptidoglycan of all Gram-negative and certain Gram-positive bacteria, can activate the sensor NOD-1. This leads to the activation of TANK Binding Kinase 1 (TBK1) and IFN- β expression (Watanabe et al., 2010).

Not only viruses or bacteria can trigger IFN-I induction. It can also be induced by fungal infections. In fact, studies show that molecules mimicking beta-glucans from candida albicans can trigger IFN- β induction in DC via activation of Dectine-1 (del Fresno et al., 2013).

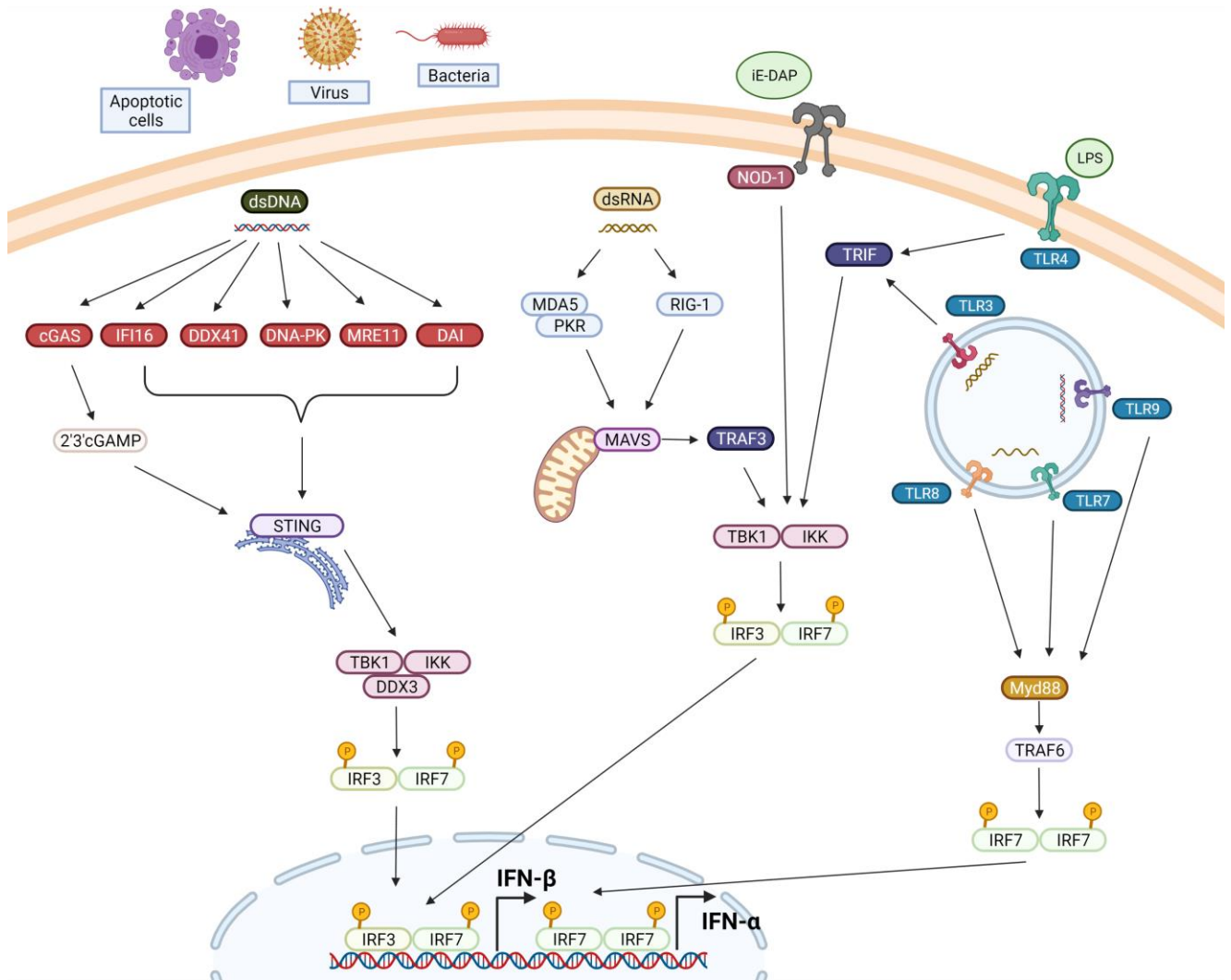


Figure 1 : IFN-I inducing signaling pathways

IFN-I is induced by several activation pathways. Viral, bacterial, or endogenous nucleic acids and components of the bacterial wall are recognized by several PRR, resulting in the expression of IFN-I. PRR includes TLR, RLR, NLR, and intracellular DNA sensors. After recognition, IRFs are phosphorylated, which leads to homo- or hetero-dimerization of IRFs, translocation to the nucleus, and transcription of IFN-I.

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1.2.2. IFN-I inducing signaling pathways

After binding to their ligands, sensors activate signalization cascades. TBK1 is recruited by many activated sensors: TLR4, TLR3, RIG-1, MDA5, IFI16, DDX41, DNA-PK, MRE11, DAI, and cGAS. TBK1 is a key protein for IFN- β downstream signaling (Ishii et al., 2006) and directly phosphorylates IFN regulatory factor 3 (IRF3) and IRF7. Activated IRF3 and IRF7 form a heterodimer that translocates to the nucleus leading to IFN-I subtype transcription.

The recruitment of TBK1 is different between TLR and other PRR. TLR signaling is dependent on either Myeloid differentiation primary response 88 (MYD88) or TIR-domain-containing adapter-inducing interferon- β (TRIF). Activated TLR3 and TLR4 recruit the adaptor protein TRIF and form the TBK1-IKKi-TRAF3 complex leading to phosphorylation of IRF3. RIG-1 and MDA5 recruit Mitochondrial antiviral-signaling protein (MAVS)-TRAF3 at the mitochondrial membrane which recruits the TBK1-IKK complex. The signaling pathway of NOD1 also implicates the recruitment of TBK1 (Watanabe et al., 2010). TLR7, TLR8, and TLR9 act differently because they do not involve TBK1 in their signalization cascade. TLR7, TLR8, and TLR9 recruit the adaptor MYD88 which recruits TRAF6 instead of TBK1. TRAF6 phosphorylates IRF7, leading to its homodimerization and translocation into the nucleus for IFN-I gene transcription. TLR4 can also activate MYD88 but IFN- β induction is only TRIF-dependent (Yamamoto et al., 2003).

Cytosolic DNA sensors do not recruit TBK1 directly but rather, via the recruitment of STING. This protein is at the mitochondrial membrane and, when activated, recruits TBK1 with DDX3 and IKKe. The activation of STING is either direct or mediated by 2'3'cGAMP, a product of the cGAS interaction with dsDNA. It can also be triggered by 3'3'cGAMP, a molecule produced by bacteria (Whiteley et al., 2019).

1.3. Mechanism of action of IFN-I

1.3.1. IFN-I receptors

IFN-I are expressed at a low level but have powerful activities. Their low level of expression makes them difficult to measure by standard experiments. Blood concentration of IFN- α has been measured in healthy subjects by a new and very sensitive method, the single-molecule array (SIMOA) (Quanterix). The median concentration is 1.6 fg/mL (Rodero et al., 2017).

IFN-I can act in a paracrine, autocrine, or systemic way. They are responsible for the production of interferon-stimulated genes (ISG) that have pleiotropic effects such as anti-viral effects, retro-control mechanisms, apoptosis, etc.

All IFN-I subtypes bind a unique heterodimeric receptor: Interferon- α/β receptor (IFNAR) 1 and 2. First, IFN-I binds one IFNAR subunit and then recruits the other receptor subunit. The ternary complex showed no direct interaction between IFNAR1 and IFNAR2, and the stoichiometry of this complex is 1:1:1 (Uzé et al., 2007). IFN-I subtypes do not have the same affinity for IFNAR1 and IFNAR2; this divergence seems to be one explanation for the different biological activities despite a unique receptor (Jaitin et al., 2006; Uzé et al., 2007). For example, IFN- α has a weaker affinity to IFNAR1 compared to IFN- β . The amount of IFNAR1 at the cell surface must be higher to activate the signaling cascade with IFN- α compared to IFN- β . Therefore, the concentration of IFNAR1 at the membrane is critical for IFN- α signaling activity (Uzé et al., 2007). A study showed that IFN- β expression induced by LPS can bind only IFNAR1 but use independent Signal transducer and activator of transcription 1 (STAT1) signaling (Weerd et al., 2013). Some ISG are highly sensitive and need a low amount of IFN-I to be expressed, in contrast to other ISG, which require a high amount of IFN-I. The first group is related to antiviral activities and the second to cell proliferation, chemokine activity, and inflammation (Piehler et al., 2012). The length of interaction between IFN-I and its receptor is also important for the diversity of biological activity responses. Antiviral activities are elicited after a few hours of IFN-I induction, while antiproliferative activities occur after a few days

(Piehler et al., 2012). More work needs to be done to understand the diversity of biological activities transduced by only one receptor for all IFN-I.

1.3.2. Signaling cascade of IFN-I activation

One of the early steps of IFN-I signaling is the endocytosis of the IFNAR1-IFN-IFNAR2 ternary complex (Marchetti et al., 2006). IFNAR1 and IFNAR2 are associated with two cytoplasmic tyrosine kinases (TYK): TYK2 and Janus kinase 1 (JAK1), respectively. TYK2 is implicated in the stabilization of IFNAR1 at the membrane (Ragimbeau et al., 2003). During the formation of the IFNAR1-IFN-IFNAR2 complex, TYK2 and JAK1 get closer and can trans-phosphorylate each other (Cohen et al., 1995). This leads to the recruitment of STAT1 and STAT2 by phosphorylation. The endocytosis of IFNAR1-IFN-IFNAR2 is necessary for STAT1 phosphorylation (Marchetti et al., 2006). The two activated STAT recruit IRF9 to form the ISGF3 complex (Fu et al., 1992), which migrates to the nucleus and binds the IFN-stimulated response element (ISRE) within the promoter of ISG.

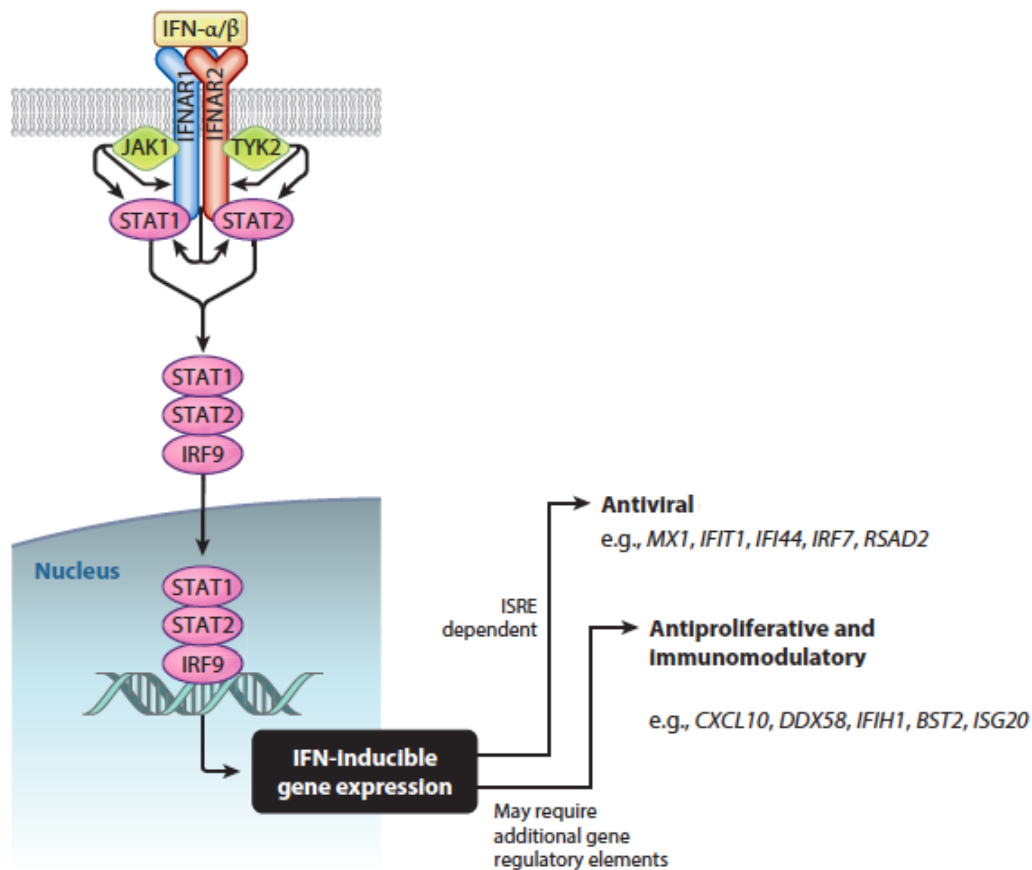


Figure 2: IFN-I signaling

The binding of IFN-I subtypes to their unique receptor induces a cascade of phosphorylation and recruitment of proteins. This signaling is responsible for ISG expression, mostly antiviral but also immunomodulatory.

Modified from (Crow et al., 2019)

1.3.3. Role of IFN-I in innate and adaptative immunity

IFN-I signaling induces the expression of more than 300 ISG that mediate the action of IFN-I (Der et al., 1998; Veer et al., 2001). Approximately 10% of the human genome is subject to IFN-I regulation (Schoggins, 2019). Among this 300 ISG, 50 are clearly involved in antiviral activities while others are implicated in inflammation, immunomodulating, signaling, and transcription. de Veer et al. show the diversity of ISG and classify them into different categories such as cell-cell adhesion, host defence, nucleotide metabolism, antiviral, apoptosis, signaling, transcription factors, and immune modulation (Veer et al., 2001). Antiviral activities of ISG can act at all levels of viral infection: entry and

trafficking, viral protein synthesis and genome amplification, and viral particle assembly and egress. Unexpectedly, a small class of ISG also promotes viral infection but their existence is not well explained yet (Schoggins, 2019).

The first described activities on immunoregulation of IFN-I were their abilities to regulate natural killer (NK) cell activities. IFN- α plays a potent role in NK cell-mediated toxicity by upregulating perforin and Fas-L (Liang et al., 2003). IFN-I, by inducing a chemokine expression, recruit NK cells to the infection site and promote the antiviral function of those cells. Moreover, IFN-I is implicated in IFN- γ production by NK cells (Zhu et al., 2008).

Also, ISG induced by IFN-I can downregulate anti-apoptotic proteins and upregulate pro-apoptotic ones in malignant cells (Chawla-Sarkar, 2003). *In vitro*, IFN- β has the property to induce the cytokines/chemokines CX3CL1 (also known as fractalkine) and CCL5 (RANTES) by vascular endothelial cells, permitting leucocyte adhesion and invasion to the infected site (Nakano et al., 2012). *In vivo*, IFN-I can also induce the release of some chemokines such as CCL2 and CXCL11, which recruit monocytes, memory T cells, and DC at the site of infection (Cepok et al., 2009).

Moreover, IFN-I has an anti-proliferative role and is at the interface between innate and adaptive immunity. IFN-I influences pDC by inducing antigen presentation and exacerbates their capacity for migration toward naive T cells. Some ISGs are part of the proteasome and are also responsible for antigen processing for the major histocompatibility complex class I (MHC-I) (Lindahl et al., 1976).

IFN-I also has an impact on adaptive immune cells, i.e. T and B cells. It plays a double role in B cell response. IFN-I has a negative effect on immature B cells because it inhibits maturation and survival (Lin et al., 1998) but has a positive effect on mature B cell activation, antibody production, and isotyping switching (Le Bon et al., 2001). Additionally, IFN-I prevents the death of the activated T cell population (Marrack et al., 1999). In response to viral infection, CD8⁺ T cells require IFN-I signaling to generate effector and memory cells (Kolumam et al., 2005).

1.4. Retro-control mechanisms of IFN-I signaling

Initially, IFN-I induces an anti-viral response but after the infection is resolved, IFN-I dampens its anti-viral response by several mechanisms. To avoid chronic IFN production, IFN-I signaling is tightly controlled.

1.4.1. Suppression of IFN-I signaling by proteins

A major mechanism for reducing IFN-I signaling is to decrease the availability of IFN-I receptors by internalization. After IFN-I stimulation, there is a decrease in IFNAR1 and IFNAR2 at the cell surface through the enhancement of ubiquitination and degradation of those receptors (Piehler et al., 2012). This process seems to affect IFN- α signaling more as compared to IFN- β . The difference could explain why long-term signaling is possible for IFN- β but not for IFN- α 2 (Uzé et al., 2007). There are two types of IFN-I inhibitors: constitutively expressed proteins that act as an activation threshold for IFN-I and induced proteins that are expressed upon infection and in response to IFN-I signaling to dampen the reaction. Three major proteins are important in the feedback loop of IFN-I signaling: Suppressors of cytokine signaling (SOCS) proteins SOCS1 and SOCS3, and the Ubiquitin specific peptidase 18 (USP18), all upregulated by IFN-I. SOCS proteins play a role in the inhibition of JAK/STAT signaling. They inhibit JAK kinase activities by their kinase inhibitory region (KIR), and SOCS1 specifically regulates IFNAR1 by interacting with TYK2, preventing its binding with IFNAR1 (Piganis et al., 2011). Because TYK2 is also involved in the stabilization of IFNAR1 at the membrane, SOCS1 is implicated in the decrease in IFNAR1 at the cell surface. USP18 acts as a suppressor by binding IFNAR2 and competes with JAK2 (Malakhova et al., 2006). Thus, it inhibits the IFN-I signaling cascade. USP18 seems to specifically inactivate IFN- α response over IFN- β (François-Newton et al., 2011) perhaps because IFN- β can induce a signal only with IFNAR1 (Weerd et al., 2013).

1.4.2. Retro-control of IFN-I signaling by microRNA

MicroRNA (miRNA) are small non-coding RNA targeting messenger RNA resulting in the cleavage, transcript destabilization, or degradation of this RNA. For maturation, primary miRNA must be

processed by mainly two enzymes: DROSHA and DICER. At first, miRNA were characterized only for cell differentiation and homeostasis but it became obvious that miRNA play a role in the immune response. Forster et al. specifically reviewed miRNA implicated in IFN-I up- or down-regulation signalization (Forster et al., 2015).

miRNA can directly target IFN- α and IFN- β transcripts or proteins implicated in IFN-I signalization. miR-466 targets specifically IFN- α mRNA whereas miR-26a, miR-34a, and let-7b target IFN- β mRNA. miRNA can also act on IFN-I signaling cascade modulate IFN-I. In mice, miR-29a has been implicated in the reduced expression of IFNAR1 on the surface of thymic epithelial cells (TEC). Loss of this miRNA induces upregulation of IFNAR1, leading to hypersensitivity to PAMP and driving thymic involution (Papadopoulou et al., 2012).

By investigating miRNA abnormalities in systemic lupus erythematosus (SLE), a disease linked to IFN-I overexpression, Tang et al. discovered that miR-146 was decreased (Tang et al., 2009). miR-146 is implicated in the downregulation of STAT1, decreasing its availability for IFN-I signalization. Indeed, mice lacking miR-146 develop autoimmune diseases, showing the importance of miR-146.

Besides miR-146, miR-221/222 and miR-145 are also implicated in STAT1 and STAT2 regulation (Zhang et al., 2010). These miRNA blocked IFN-I signalization by diminished STAT expression. miR-373 diminished the expression of JAK1 and IRF9, leading to lower IFN-I anti-viral signaling (Mukherjee et al., 2015).

Few miRNA can also target proteins implicated in the retro-control of IFN-I. It has been demonstrated in mice and in humans that miR-155, which is induced by IFN-I, inhibits SOCS1 and, therefore, enhances IFN-I response (Pathak et al., 2015; Wang et al., 2010). miRNA can target not only IFN-I signaling but also proteins involved in IFN-I production. TLR2, TLR4, and MYD88 have been shown to be regulated by miR-19 (Philippe et al., 2012), let7e, and miR-200 (Wendlandt et al., 2012), respectively.

Regulatory miRNA play a major role at all levels in the IFN-I machinery, and deregulation could lead to several pathologies.

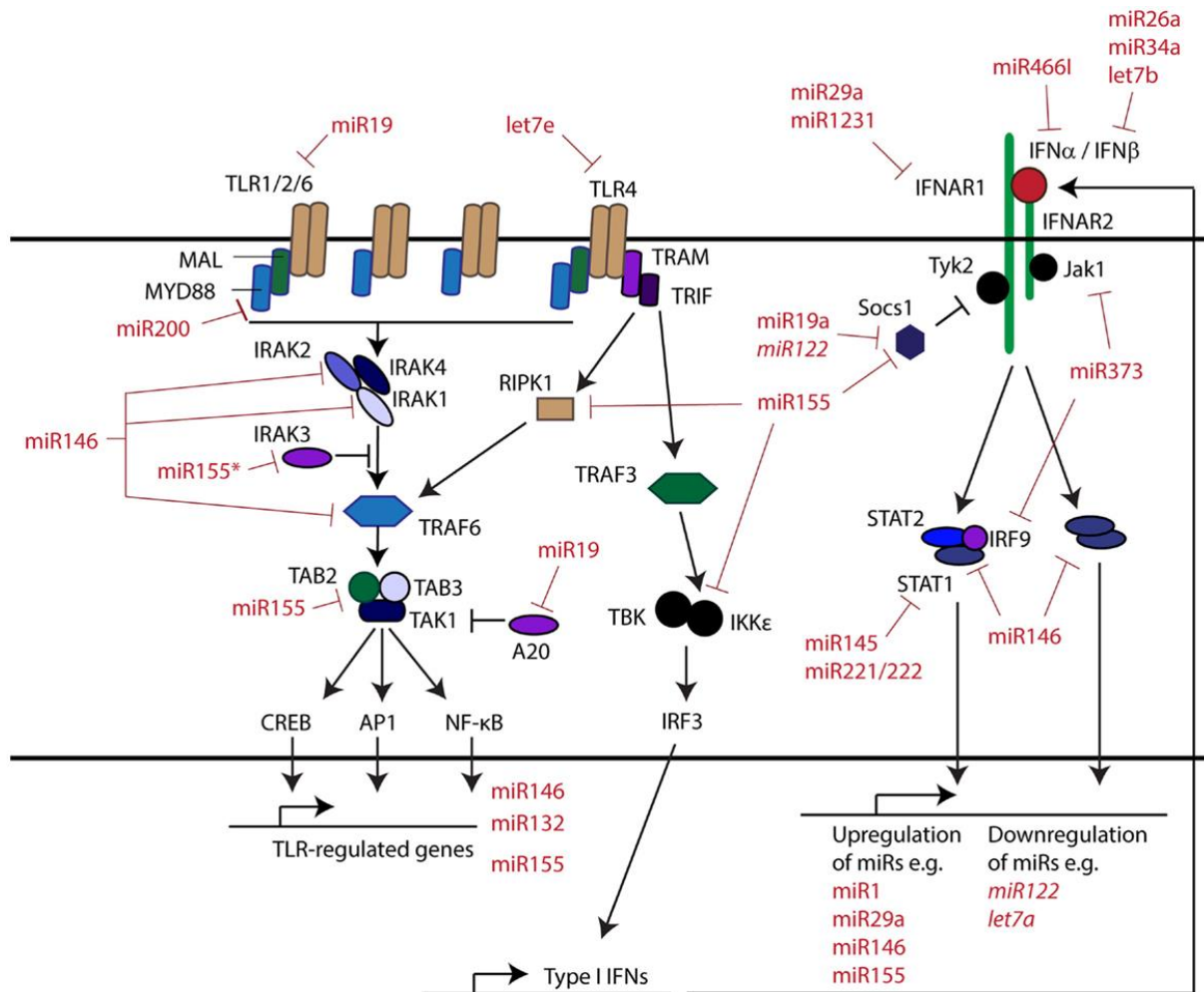


Figure 3: miRNA regulation of IFN-I signaling

miRNA play an important role in the regulation of gene transcript by degrading mRNA. Several miRNA are implicated in IFN-I signaling at different levels.

Modified from (Borden et al., 2007)

1.5. Implication of IFN-I in diseases

1.5.1. Type I interferonopathies: Genetic diseases

In 2011, Crow et al. defined a new group of diseases with the same feature, i.e., autoinflammation and overexpression of IFN-I: the Interferonopathies (Crow, 2011). They are genetic diseases with childhood-onset, and 18 mutated genes have been identified in patients so far (see table 1). All these genes are involved in IFN-I production or signaling. Patients present an IFN-I signature in the periphery and also inflammation.

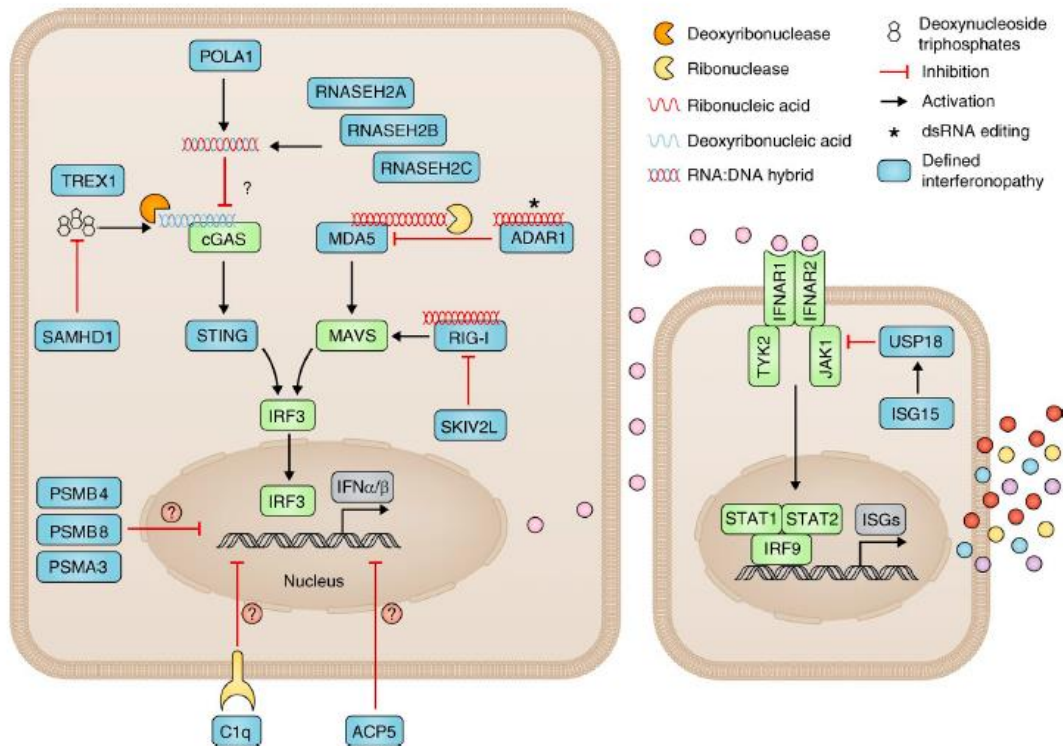


Figure 4: Mutated genes involved in type I interferonopathies

Mutated genes implicated in interferonopathies are linked to IFN-I signaling: In the degradation of nucleic acids, in the negative feedback of IFN-I, and in the expression of ISG. Molecules implicated in interferonopathies are represented in blue boxes.

From (Rodero and Crow, 2016)

The prototypic interferonopathy is Aicardi–Goutières syndrome (AGS), a genetically heterogeneous disease with diverse gene mutation, all linked to IFN-I signaling (Aicardi and Goutières, 1984). AGS is characterized by a constitutive IFN-I expression in response to endogenous nucleic acids. IFN- α is a consistent marker of AGS, as it can be found in cerebrospinal fluid and sera (Lebon et al., 1988). Even if IFN- α tends to decrease during the course of the disease, ISGs are still upregulated in the PBMC of patients (Rice et al., 2013). IFN- α seems to be the key feature of the disease because there are only antibodies against IFN- α and not against IFN- β . However, no suitable test for the detection of IFN- β was available at the time of the disease characterization (Lebon et al., 2002). The seven

potentially mutated genes associated with AGS are linked to either nucleic acid metabolism (TREX1, RNaseH2A, RNaseH2B, RNaseH2C, SAMDH1, or ADAR) or IFN-I signaling (IFIH1) (Crow, 2015). Genes implicated in nucleic acid metabolism are mostly nuclease or editing enzymes.

Seven other diseases compose the interferonopathy family:

- Familial chilblain lupus shares some gene mutations with AGS on TREX1, SAMDHD1, and TMEM173. It is a monogenic form of cutaneous lupus with early onset characterized by ISG in PMBC.
- Retinal vasculopathy with cerebral leukodystrophy (RVCL) is a disorder characterized by an autosomal dominant mutation of TREX1 with onset in adolescence. Patients showed a heterozygous TREX1 mutation with an IFN-I signature in blood and inflammatory processes.
- Spondyloenchondrodysplasia (SPENCD), a skeletal dysplasia, presents a biallelic mutation of the ACP5 gene responsible for the TRAP protein. Its role is to hydrolyze different proteins such as osteopontin. This protein promotes activation of IFN- α in pDC. TRAP is also implicated in the dephosphorylation of nucleic acids that might activate sensors such as RIG-1. Mutation in this gene can lead to an abnormal expression of IFN- α , typically found in SPENCD patients.
- STING associated vasculopathy (SAVI) with onset in infancy is a disease with a *de novo* mutation on the TMEM173 genes coding for STING protein. It is a gain of function mutation and, therefore, a constitutive activation of STING.
- C1q deficiency can also be part of the interferonopathy family. This is a disease caused by a biallelic loss of function of the C1q protein. It is linked at 90% with SLE. Studies show that, normally, C1q inhibits IFN- α expression by pDC in response to RNA-associated immune complex and CpG (Lood et al., 2009).
- Single Merton syndrome is characterized by skeletal abnormalities with early-onset. It is an inherited syndrome with an autosomal dominant manner caused by mutation gain of function for IFH1 and RIG-1 genes. This leads to constitutive IFN-I activation.

- USP18 deficiency clinical features resemble AGS and are characterized by IFN-I activation. USP18 protein is involved in the retro-control of IFN-I signaling.

Mechanisms of monogenic type I IFN-mediated disorders			
Mechanism of disease	Gene	Protein function	Disorder
Abnormal accumulation of nucleic acids	<i>TREX1</i>	Three prime repair exonuclease 1; cytosolic DNase	Aicardi-Goutières syndrome (AGS), Familial chilblain lupus (CHBL), Retinal vasculopathy with cerebral leukodystrophy (RVCL)
	<i>RNASEH2A</i>	Ribonuclease H2, subunits A, B, C; ribonucleotide excision repair	Aicardi-Goutières syndrome (AGS)
	<i>RNASEH2B</i>		
	<i>RNASEH2C</i>		
	<i>SAMHD1</i>	SAM domain and HD domain-containing protein 1; dNTP triphosphohydrolase, RNase	Aicardi-Goutières syndrome (AGS), Familial chilblain lupus (CHBL)
Abnormal chemical modification of nucleic acids	<i>POLA1</i>	DNA polymerase α ; synthesis of RNA-DNA primer during DNA replication	X-linked reticulate pigmentary disorder (XLRPD)
	<i>ADAR1</i>	Adenosine deaminase, RNA-specific; deamination of adenosine to inosine in dsRNA	Aicardi-Goutières syndrome (AGS)
Enhanced sensitivity or ligand-independent activation of nucleic acid sensing pathways	<i>TMEM173</i>	Stimulator of interferon genes protein; IFN- β induction in response to cytosolic DNA	STING-associated vasculopathy, infantile-onset (SAVI), Familial chilblain lupus (CHBL)
	<i>IFIH1</i>	IFN-induced helicase C domain-containing protein 1; pattern recognition receptor for dsRNA	Aicardi-Goutières syndrome (AGS), Singleton-Merten syndrome (SGMRT)
	<i>DDX58</i>	Retinoic acid-inducible gene 1 protein; pattern recognition receptor for dsRNA	Singleton-Merten syndrome (SGMRT)
Impaired negative regulation of nucleic acid-induced type I IFN signaling	<i>ISG15</i>	Interferon-stimulated gene 15; ubiquitin-like protein, modifies proteins by ISGylation	ISG15 deficiency
	<i>USP18</i>	Ubiquitin specific peptidase 18; de-ISGylation	USP18 deficiency
	<i>ACP5</i>	Tartrate-resistant acid phosphatase, type 5; dephosphorylation of osteopontin	Spondyloenchondrodysplasia (SPENCD)
Modulation of type I IFN responses independent of nucleic acid sensing	<i>PSMB8</i>	Proteasome subunit beta type-8; antigen processing in immunoproteasome	Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE)
	<i>PSMB4</i>	Proteasome subunit beta type-4; antigen processing in immunoproteasome	
	<i>PSMA3</i>	Proteasome subunit alpha type-3; antigen processing in immunoproteasome	
	<i>PSMB9</i>	Proteasome subunit beta type-9; antigen processing in immunoproteasome	
	<i>POMP</i>	Proteasome maturation protein; formation of immunoproteasome	

Table 1: Mutated genes implicated in interferonopathies

From (Kretschmer and Lee-Kirsch, 2017)

1.5.2. Acquired interferonopathies: Autoimmune diseases

Some non-genetic autoimmune diseases are also considered interferonopathies. Systemic lupus erythematosus (SLE) is a rare autoimmune disease characterized partially by the presence of anti-nuclear antibodies. It is a multisystemic disease with multiple organ manifestations, various genetic mutations, and environmental factors. Many genetic polymorphisms are linked to the innate or adaptative immune system. Patients with SLE have a loss of immune tolerance, which leads to autoantibodies with accumulation and deposition of immune complex in tissues. Those immune

complex lead to inflammation, which is the main feature of this disease. SLE was the first disease suspected to be caused by IFN-I. In fact, an IFN-I signature is found in the blood of almost all children with SLE and 50% of adult patients. Various evidence has demonstrated the involvement of IFN-I in SLE: circulation immune complex can induce IFN production and polymorphism in the IFN-I pathway has been identified in a GWAS (Cui et al., 2013).

Patients with juvenile-onset dermatomyositis, an autoimmune disease characterized by weakness, skin rashes, and muscle inflammation, present an overexpression of IFN- α in serum and an IFN-I signature in muscle and skin (Rodero et al., 2017). Moreover, the expression of ISG is correlated with the disease activity but the source of IFN-I remains unknown (Greenberg et al., 2005). It appears that IFN-I plays a major role in several autoimmune diseases.

1.5.3. Iatrogenic effect of IFN-I

IFN-I is also used as a treatment for various diseases and can induce transient autoimmune diseases in some patients. Several publications showed that IFN-I immunotherapies can induce SLE (Bahri et al., 2012). The clinical onset can be from weeks to years. After the discontinuing of IFN-I treatment, patients stop displaying SLE symptoms (Nousari et al., 1998).

IFN- α is used as a treatment in the case of chronic hepatitis C viral infection and some patients develop myasthenia gravis (MG) symptoms. Baik et al. published a history of all MG cases induced by IFN- α used as a treatment for chronic hepatitis C (Baik et al., 2016). Those patients displayed different features of MG such as autoantibodies against α -AChR, muscle weakness, and the more severe symptom of this disease: respiratory crisis. In case of the appearance apparition of MG-like symptoms, patients were given traditional treatment for MG.

IFN- β can also be given as a treatment for patients with multiple sclerosis (MS). Two case reports showed the development of MG following treatment with IFN- β (Dionisiotis et al., 2004).

2. Myasthenia Gravis

2.1. Generalities

Myasthenia Gravis (MG) is a rare autoimmune disease with a prevalence of 150-250 cases per million individuals (Carr et al., 2010). MG is a multifactorial disease resulting from a combination of genetic predisposition and environmental risk factors. The disease can be classified according to the severity of the symptoms, age of onset, antigenic targets, and thymic-associated abnormalities. Early-onset MG (EOMG) patients declare symptoms before the age of 45-50 years old. Late-onset MG patients declare the disease after 45-50 years old; these cases are often associated with thymoma. Very late-onset MG patients declare the disease after 70 years old. These subgroups display different features with female predominance for EOMG and an equal sex ratio for late-onset MG and very late-onset MG. The female predominance in EOMG and other autoimmune diseases could be linked to sex hormones (Moulton, 2018). Indeed, estrogen downregulate AIRE expression in the thymus and reduce the tolerance process during T cell education (Dragin et al., 2016). Low testosterone in males could be associated with a higher risk of developing MG (Pakpoor et al., 2016). MG is characterized by autoantibodies targeting post-synaptic components at the neuromuscular junction, leading to a neurotransmission defect.

Genetic predisposition

Though MG is not a genetic disease, some genetic predispositions have been linked to it. Variants of HLA genes, molecules implicated in antigen presentation, have been found in EOMG patients, especially the variant HLA-DR3-B8 (Giraud et al., 2008). A specific genetic predisposition for Muscle-specific kinase (MuSK)-MG patients has been discovered to be associated with the variant HLA-DQ5 (Marino et al., 2014). Other non-HLA-related genes are also associated with MG. Interestingly, a variant in the promoter of α -acetylcholine receptor (AChR), the main target antigen of MG, is associated with EOMG (Giraud et al., 2007). This could explain the sensitivity to this antigen.

2.1.1. Symptoms of MG patients

The main symptom of MG is muscle weakness involving different muscles. Symptoms are more severe throughout the day, with physical activity and heat. In the short term, no damage to the muscle is found but in the long term, some patients who do not respond to treatment can display muscle damage. MG is considered a fluctuating disease because symptoms can vary but there is no continuous progression of the disease (Grob et al., 2008).

Based on symptoms, two major forms of the disease are described: an ocular form (15%) and a generalized form (85%). The Myasthenia Gravis Foundation of America divided patients into five main classes depending on the severity of symptoms.

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

Table 2: Clinical MGFA classification of MG patients

From (Heldal et al., 2014)

Symptoms of the ocular form are usually the initial signs of the disease. The two main symptoms are ptosis (drop of the upper eyelid) and diplopia (double vision) (Nair et al., 2014). Half of the patients presenting the long-lasting ocular form have anti-AChR antibodies. Over the first 2 years after diagnosis, around 80% of MG patients evolve from an ocular to a generalized form (Grob et al., 2008). Generalized MG involves bulbar, limb, facial, and respiratory muscle. A respiratory crisis is the

main life-threatening event that occurs in MG patients and leads to hospitalization. It can be triggered by diverse reasons like infection, emotional stress, surgeries, or medication. Spontaneous remissions are uncommon and usually associated with an ocular or mild generalized MG with juvenile-onset (Mano and Takegami, 1993).

MG patients with anti-MuSK antibodies do not present the exact same symptoms as AChR-MG patients. They present fewer ocular symptoms and usually have more severe symptoms with bulbar muscle impairment such as dysarthria, dysphagia, facial weakness, and swallowing difficulties (Evoli et al., 2003). Lipoprotein related 4(LRP4)-MG patients usually have mild symptoms as compared to AChR-MG and MuSK-MG (Zisimopoulou et al., 2014).

2.1.2. Antigenic targets in myasthenia gravis

Myasthenia Gravis is an autoimmune disease with autoantibodies against components of the neuromuscular junction.

2.1.2.1. Description of the neuromuscular junction

The neuromuscular junction (NMJ) is a synapse between a motor neuron and a muscle fiber. NMJ is composed of a presynaptic terminal with the motor neuron, the postsynaptic muscle membrane, and the space between, called the synaptic cleft. Different proteins are found at the muscle membrane, such as MuSK, LRP4, and AChR. MuSK and LRP4 play an important role in the formation of the NMJ and the clustering of AChR. Secreted agrin by the motor neuron binds LRP4, which, in turn, binds MuSK, resulting in its activation and phosphorylation (Zhang et al., 2011). Phosphorylated MuSK recruits different adaptor proteins necessary for AChR clusterization. AChR is present at the top of the fold of post-synaptic membranes, forming a canal composed of five subunits: 2α , 1β , 1δ , and 1ϵ (in adult) or 1γ (in embryo).

The presynaptic terminal of the neuron contains vesicles filled with acetylcholine. An electric signal in the neuron, called action potential, induces the exocytosis of the acetylcholine in the NJM. The binding of ACh to the α subunits of AChR induces a conformational change to an open canal, leading

to an influx of Na^+ inside the muscle cell (Miyazawa et al., 2003). This influx induces depolarization of the motor plaque, resulting in contraction of the muscle. To terminate synaptic transmission, an enzyme called acetylcholinesterase hydrolyzed ACh in the synaptic cleft.

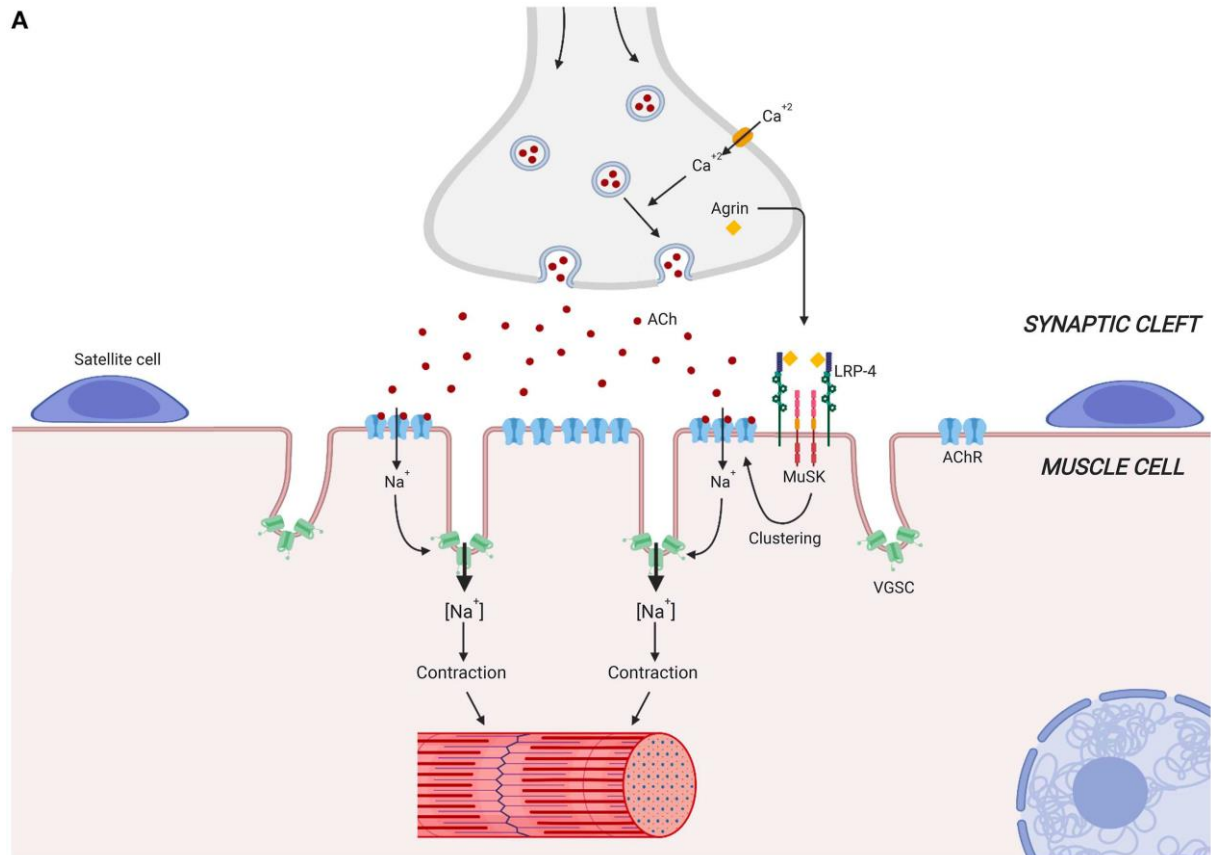


Figure 5: Structure of the neuromuscular junction

The action potential of the motor neuron induces the release of acetylcholine (ACh) in the synaptic cleft. ACh then binds the ACh receptor (AChR) at the membrane of muscle, inducing conformation changes of the receptor into an open pore. This leads to an influx of Na^+ in the muscle, resulting in muscle contraction.

From (Vilquin et al., 2019)

2.1.2.2. MG with anti-AChR antibodies

The first discovered pathogenic autoantibodies were against the AChR (Aharonov et al., 1975). Anti-AChR antibodies represent 85-90% of MG patients. Anti-AChR is found in EOMG and LOMG patients and is usually associated with thymic hyperplasia or thymoma, respectively. The main immunogenic

region (MIR) of AChR is on the α subunits and, therefore, antibodies against this region are the most pathogenic (Tzartos et al., 1998). Most generalized-MG patients present antibodies against the MIR of AChR, which explains the more severe symptoms. Anti-AChR antibodies are polyclonal and mostly against conformational epitope. The major subtypes of autoantibodies are IgG1 and IgG3 and, at a lower concentration, IgG2 (Fichtner et al., 2020). The pathogenicity of IgG1 and IgG3 anti-AChR antibodies works in different pathways.

- Complement pathway (Engel et al., 1981): Complement activation by deposition of IgG1 or IgG3 is the main pathway of neuromuscular junction degradation. It leads to the formation of the membrane attack complex that induces post-synaptic damage and destruction. Destruction induces a decrease in the number of AChR at the membrane surface and increases the threshold for muscular contraction (Ruff and Lennon, 2008).
- Antigenic modulation: Anti-AChR antibodies bind AChR and accelerate their internalization at the membrane surface by endocytosis, leading to their destruction (Heinemann et al., 1977).
- Blocking of the receptor: By binding the receptor, anti-AChR antibodies prevent the binding of ACh with its receptor and, therefore, its activation. This pathway is not the most frequent (Almon et al., 1974).

Though the symptoms are due to autoantibodies, anti-AChR antibody titer is not linked to the severity of the disease (Limburg et al., 1983). To compensate for the loss of AChR at the postsynaptic membrane, muscle cells increase AChR subunit expression but this does not fully compensate for the destruction (Guyon et al., 1994).

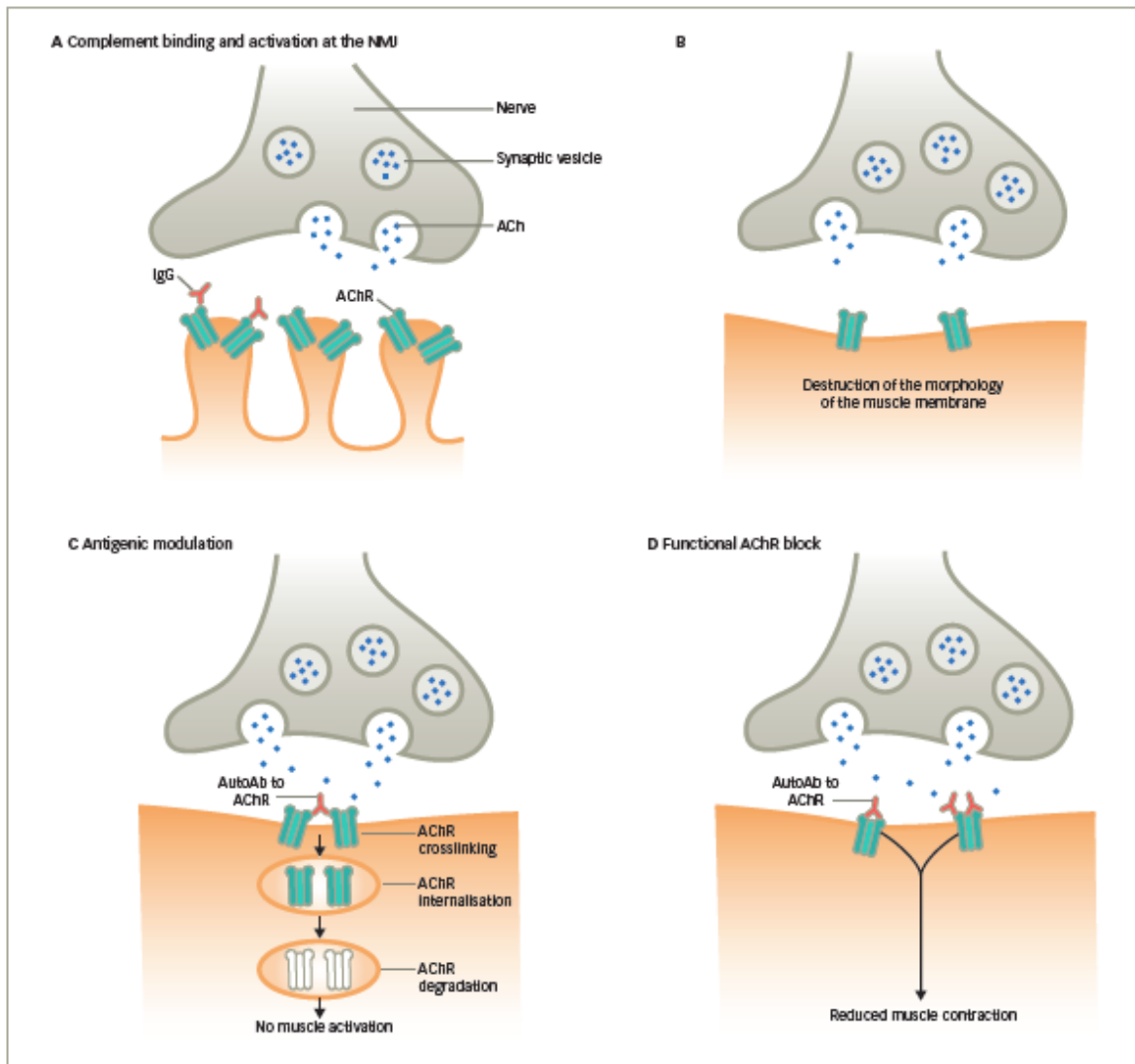


Figure 6: Pathological action of autoantibodies in AChR MG patients

Antibodies can act in three different ways: (A-B) activation of complement and destruction of the NMJ, (C) internalization of AChR reducing the availability for ACh binding, and (D) blocking of AChR.

From (Jacob and Queen Elizabeth Neuroscience Centre, University Hospitals of Birmingham NHS

Foundation Trust, Birmingham, UK, 2018)

2.1.2.3. MG with anti-MuSK antibodies

For a long time, 20% of MG patients were considered seronegative. In 2001, autoantibodies against MuSK were discovered in 70% of so-called seronegative MG patients (Hoch et al., 2001). MuSK-MG patients are more prone to severe and generalized muscle weakness. Anti-MuSK antibodies are mostly IgG4 and, therefore, do not activate the complement cascade and do not lead to AChR

internalization. Their major role is to block the interaction between MuSK and LRP4 inhibiting the clusterization of AChR. On the contrary to anti-AChR antibodies, the anti-MuSK antibody titer is correlated with the severity of the disease (Niks et al., 2008).

2.1.2.4. MG with anti-LRP4 antibodies

Even more recently, autoantibodies targeting LRP4 have been discovered in AChR and MuSK seronegative MG patients (Higuchi et al., 2011). Patients with anti-LRP4 present ocular or generalized mild MG, usually observed in young women. Anti-LRP4 antibodies block the interaction between agrin and LRP4; therefore, they inhibit the activation of MuSK, which interferes with the agrin-induced AChR clusterization and destabilizes the NJM (Shen et al., 2013). As for anti-AChR antibodies, anti-LRP4 antibodies are mostly IgG1 and IgG2 isotypes (Zisimopoulou et al., 2014). They act as antigenic modulation by accelerating endocytosis of LRP4 and, thus, diminish agrin activities but also activate the complement cascade leading to degradation of the post-synaptic membrane (Shen et al., 2013).

Anti-LRP4 antibodies are also occasionally found in 8% of AChR-MG patients, 15% of MuSK-MG patients, and 4% of other neurological diseases (Zisimopoulou et al., 2014).

2.1.2.5. Other autoantibodies implicated in MG

Other autoantibodies are found in MG patients targeting agrin, titin, ryanodine receptors, rapsyn, cortactin, collagen Q, and collagen XII (Lazaridis and Tzartos, 2020). However, their pathogenic role is not defined yet. They usually co-exist with one of the three main autoantibodies involved in MG.

Two targeted proteins are present in the NJM: agrin and collagen Q. Agrin plays a major role in the NJM, and anti-agrin antibodies are found in MG patients regardless of their major antibodies, anti-AChR, anti-MuSK, or anti-LRP4. In fact, immunized mice with agrin showed anti-agrin antibodies and MG-associated symptoms, showing a potential role of agrin in MG pathogenesis (Yan et al., 2018). Collagen Q is important for the anchoring of acetylcholinesterase in the NJM. The pathogenicity of Collagen Q and XIII antibodies remains unclear.

Cytoplasmic muscle proteins can also be targeted by autoantibodies. Cortactin is involved in the AChR clustering induced by MuSK, and anti-cortactin antibodies are found in seronegative and seropositive patients (Gallardo et al., 2014). These autoantibodies are also found in other autoimmune diseases, especially myositis-associated. Titin is a key protein for muscular contraction, and anti-titin antibodies are found in 20-30% of MG patients with AChR antibodies, especially LOMG patients and thymoma-associated MG (MGT) patients (Szczudlik et al., 2014). Patients with anti-titin and anti-ryanodine present a more severe form of MG.

2.1.3. Treatments for MG patients

Different treatments are available for MG patients but they only treat the symptoms or correspond to non-specific immunosuppressors. One of the first treatments used was acetylcholinesterase inhibitor, in 1934 (Bodman, 1934). This treatment increases the bioavailability of ACh at the NMJ and increases the chance that ACh interacts with its receptor. MG patients usually respond well to this treatment, except for MuSK-MG patients, for whom it can exacerbate muscle weakness (Evoli et al., 2003). Sometimes, AChEI on its own is not sufficient, so immunomodulatory treatments are needed. Corticosteroid is the first line of immunotherapy treatment used for MG patients, and 74% of patients are good responders. Immunosuppressors can also be added but take a long time to become effective, in contrast to corticosteroids. Those treatments work mainly by dampening the immune system. All types of MG, especially MuSK-MG, respond well to those treatments. However, both corticosteroids and immunosuppressors have many secondary effects (Gilhus et al., 2019).

During an MG crisis, intravenous immunoglobulin (IVIg) or plasma exchange can be used because of their rapid efficacy but they are short-term treatments. IVIg consists of the injection of a high dose of non-specific immunoglobulins that act by inhibiting the complement, blocking the Fc receptor, and neutralizing cytokines and antibodies (Gelfand, 2012). Meanwhile, plasma exchange works by removing antibodies, complement components and cytokines.

Surgical treatment can be proposed for MG patients: thymectomy. It is recommended only for anti-AChR EOMG or thymoma-associated MG patients. A clinical trial on AChR MG patients (not including

thymoma patients) showed that thymectomy is beneficial especially for patients under 50 years old as compared to older patients. Thymectomy with prednisolone showed an improvement of symptoms in MG patients compared to prednisolone alone (Berrih-Aknin and Le Panse, 2019; Wolfe et al., 2016). Some reports showed that incomplete thymic removal leads to less efficacy of thymectomy with regard to MG symptoms. Improvement of patients with thymectomy supports the evidence that the thymus plays a central role in EOMG.

Despite the well-known efficacy of these classical treatments, 10-30% of patients do not respond to them; these patients are called “refractory patients.” Usually, patients are MGT or anti-MuSK patients. In these cases, rituximab and eculizumab have proven their efficacy. Eculizumab is an antibody directed against the complement component C5 and is indicated for AChR-MG patients (Jacob and Queen Elizabeth Neuroscience Centre, University Hospitals of Birmingham NHS Foundation Trust, Birmingham, UK, 2018). This treatment is expensive and not used worldwide yet. However, there is no consensus about the ideal MG treatment.

Treatments Main medications	Efficiency in:				General information	
	AChR-MG	Clustered AChR-MG	MuSK-MG	LRP4-MG	Mechanism of action	Main adverse side effects
AChE inhibitors Mestinon Mytelase Neostigmine Pyridostigmine	Yes	Yes	No	Yes	Block the enzyme that degrade ACh and prolong the half-life of ACh	Minor side effects: stomach cramps, nausea, gut hyperactivity, diarrhea...
Corticosteroids Prednisolone	Yes	Yes	Yes	Yes	Weakened the immune system	Can have severe side effects mainly: osteoporosis, weight gain, decreased resistance to infection, high blood pressure, increased susceptibility to diabetes...
Immunosuppressors Azathioprine Cyclosporine Mycophenolate Mofetil	Yes	Yes	Yes	Yes	Block nucleotide synthesis and lymphocyte proliferation Lowered corticosteroid requirement	Divers according to the medication used but can have severe side effects. Careful medical follow-up needed
B-cell depletion Rituximab	?	?	Yes	?	CD20 antibody that reduce the number of circulating B cells	Do not target plasma B cells
Complement inhibitors Eculizumab	Yes	?	No	?	Anti-C5 antibody preventing the formation of the membrane attack complex	headache, dizziness, fever, nausea, infections, fatigue
Broad anti-immune therapies Plasma exchange immunoadsorption IvIGs	Yes	Yes	Yes	Yes	Reduction of pathogenic autoantibodies. Applied during crisis	Minor side effects
Thymectomy	Yes	Probably	No	?	In thymoma, removal of the tumour In EOMG, reduce severity of MG	If any, especially link to the surgical procedure

Table 3: Current treatments for MG

2.2. Experimental models

Different animal models have been developed to better understand the pathology of MG.

2.2.1. Classical experimental autoimmune MG model

Inducible MG rodent models have been developed in mice and rats by injecting the autoantigen: the experimental autoimmune MG (EAMG) model (Green et al., 1975). Briefly, α -AChR purified from electric organs of torpedo fish or peptides are mixed with a strong adjuvant for a better immune response and injected in the footpads and backs of mice. Animals will start developing an immune reaction against the α -AChR antigen from torpedo, and by cross-reaction, antibodies will attack AChR from the recipient. Animals present similar symptoms as MG patients, including muscle weakness but also the presence of anti-murine AChR antibodies in their serum (Christadoss et al., 2000).

However, this experimental model fails to recapitulate thymic abnormalities specific to MG pathology.

2.2.2. Engrafted MG-NSG model

The MG-NSG model consists of the graft of small thymic fragments from MG patients in NOD scid gamma C KO (NSG) mice. This strain of mice is severely immunodeficient and, therefore, tolerates the xenograph. Grafted mice display human anti-AChR antibodies with a cross-reaction against murine AChR (Schönbeck et al., 1992). The MG-NSG model also displays MG features such as muscle weakness. Interestingly, the graft thymus is still active in the mice. They contain germinal centers (GC) and the differential gene expression linked to GC establishment is still observed (Sudres et al., 2017). This model showed the involvement of the thymus in the pathology compared to the EAMG model.

2.3. AChR-MG physiopathology

2.3.1. The thymus in all states

2.3.1.1. The normal thymus

Anatomy of the thymus

The thymus is a primary lymphoid organ and a central organ for the immune system in terms of educating T cells to discriminate self- and non-self-proteins. It is found in the anterior mediastinum, above the heart (Nishino et al., 2006). The thymus reaches its maximal weight during puberty and then undergoes involution, with size and weight reduction, during life (Lynch et al., 2009; Steinmann et al., 1985). The thymic tissue is replaced progressively by adipose tissue with aging. The thymus is divided into two main cellular zones: the cortex and the medulla, implicated in positive and negative thymocyte selections, respectively. It is composed of different stromal cells: thymic epithelial cells (TEC), fibroblasts, macrophages, dendritic cells, and myoid cells. Medullary and cortical TEC are distinct according to their differential expression of keratins: K8 and K18 for cortical TEC (cTEC) and

K5 and K14 for medullary TEC (mTEC). TEC are a major component of thymic stroma and play an important role in antigen presentation, essential for thymocyte selection. A specific thymic structure called the Hassall corpuscle is found in the medulla and correspond to an ultimate stage of differentiation of TEC (Ortiz-Hidalgo, 1992). Their functions are not well understood yet.

Myoid cells are a rare population localized at the medulla and cortico-medullary junction. They have characteristics of skeletal muscle cells with expression muscle proteins such as MyoD, desmin, troponin T, and a functional AChR (Wakkach et al., 1999). Their role is not yet well known but they seem to play a protective role for thymocytes against apoptosis (Le Panse and Berrih-Aknin, 2005). They might play a role in sensitization against AChR in MG, as they express all AChR subunits leading to a functional AChR.

Macrophages are scavenger cells with the main role of phagocytosed apoptotic cells, especially thymocytes during their selection (Platt et al., 1996). In the thymus, they are found in the medulla and cortex.

Thymic DC play a major role as antigen-presenting cells. They are important for central T cell tolerance because they induce apoptosis in autoreactive T cells and also play a role in the development of thymic regulatory T cells (Evans et al., 2008). They can be conventional DC or pDC depending on their progenitors. This last type is a great inducer of IFN-I upon viral infection.

Though the thymus is T cell organ-specific, it can also contain a specific B cell type that differs from peripheral B cells (Perera and Huang, 2015).

Role of the thymus in thymocyte selection

The main role of the thymus is T cell maturation and education by a succession of negative and positive selection steps. T cells are usually discriminated by the expression of either CD4 or CD8. T cell lymphoid progenitors, pro-thymocytes, migrate from the bone marrow toward the cortico-medullar junction of the thymus through blood vessels (Takahama, 2006). They are attracted by different chemokines such as CCL21 (Zlotoff et al., 2010).

First, double-negative thymocytes migrate to the cortex and then become double-positive (DP), meaning that T cell antigen receptor (TCR) is linked to CD4 and CD8. DP thymocytes encounter cTEC expressing MHC-I or MHC-II, loaded with random peptides. If cortical DP thymocytes recognized the MHC-I or MHC-II on cTEC with moderate affinity, they undergo positive selection and, therefore, commit to the CD4 or CD8 lineage depending on the class of the MHC. If they recognize MHC-II, they become CD4⁺ single positive (SP) thymocytes and if they recognize MHC-I, they become CD8⁺ SP thymocytes. In cases in which the DP thymocyte does not recognize any class of MHC, they undergo apoptosis, which is a process called “death by neglect” (Klein et al., 2009). During their journey, DP thymocytes receive signals for maturation into SP and migration toward the medulla. Different chemokines are responsible for this migration: CCL21, CCL22, and CXCL10 (Yarilin and Belyakov, 2004). Medullary TEC (mTEC) play an important role in the negative selection of thymocytes by expressing tissue-specific antigen (TSA), necessary for the education of T cells and avoiding autoreactivity. These TSA are peptide-loaded on MHC-II and their expressions are under the control of AIRE, FEZ2, and BLIMP1, which are important transcription factors expressed by mTEC (Roberts et al., 2017). If the TCR of SP thymocytes interacts with high avidity with the MHC loaded with self-antigen TSA, the cell is eliminated to avoid autoreactivity. This negative selection is a barrier to autoimmunity. In fact, mTEC can also express α -AChR as TSA, which is important in the case of MG. The thymic medulla has plays an important role in the induction of regulatory T cells (Treg) (Bensinger et al., 2001). Indeed, when a CD4⁺ T cell has a high affinity for MHC-II peptide antigen, it can start expressing the transcript factor FoxP3 and become a Treg. Treg plays an important role in what we call peripheral tolerance. At the end of positive and negative selection, the chemokine CCL19 promotes the exportation of mature T cells to the periphery. Only 1% of thymocytes make it through to this export (Scollay et al., 1980).

To summarize, the thymus is the place where central tolerance and depletion of autoreactive T cells are established. When auto-reactive T cells escape in the periphery, others mechanisms of peripheral tolerance can occur to suppress auto-reactivity.

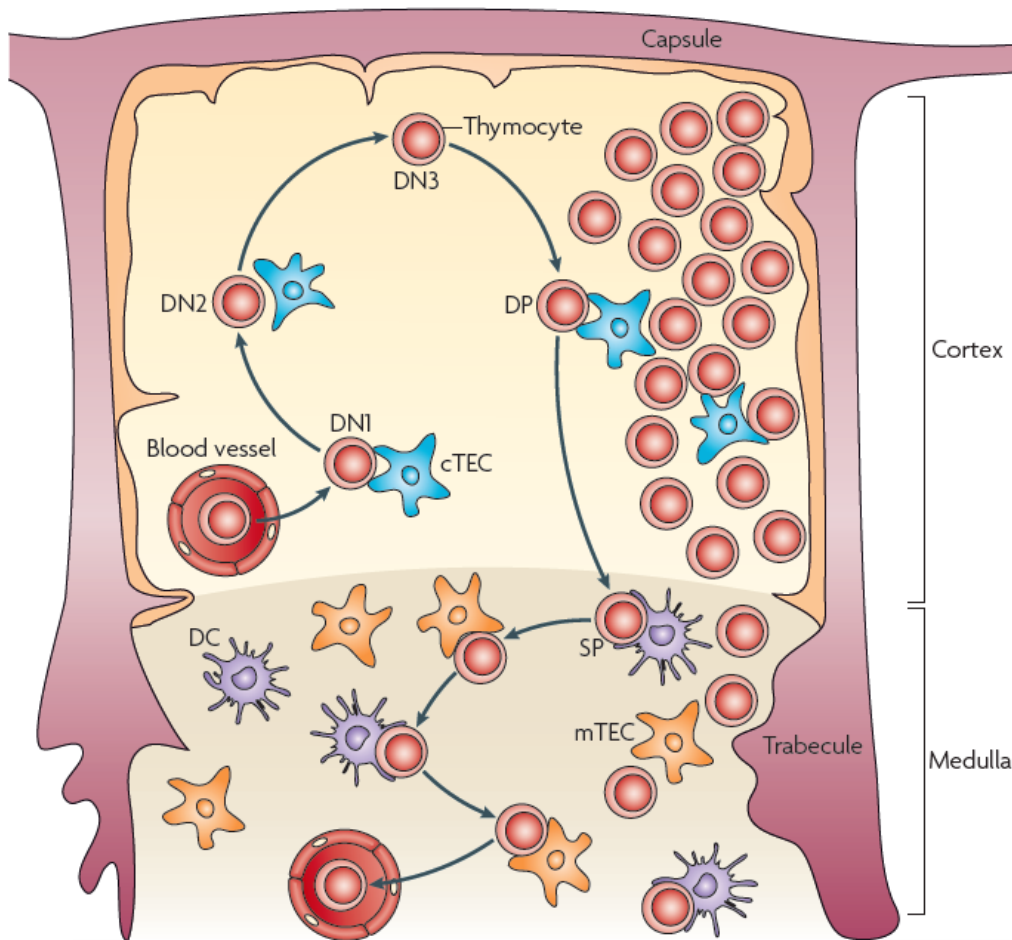


Figure 7: A thymocyte's journey through the thymus

Fresh thymocytes enter in the thymus through blood vessels at the cortico-medullary junction. Then, they become DP and met cTEC. TCR affinity with MHC-I or II determines CD4 or CD8 T cell lineage. Next, SP thymocytes are attracted to the medulla, encountering the mTEC for positive selection. This selection is important to avoid the development of autoreactive T cells. Selected thymocytes are exported out of the thymus for peripheral immunosurveillance.

From (Klein et al., 2009)

2.3.1.2. The pathological thymus

2.3.1.2.1. Thymus of EOMG-AChR patients

In AChR MG patients, if the muscle is the target organ, the thymus is the effector organ. Thymic changes are clearly observed in EOMG patients but not in LOMG, as the thymus is strongly involuted

and thymectomy does not seem to have a beneficial effect (Leite et al., 2005). The thymus is not altered in MuSK MG patients and is not clearly defined for LRP4 MG patients (Leite et al., 2005).

The EOMG thymus is characterized by B cell infiltration, and 50-60% of them display follicular hyperplasia defined by the development of ectopic GC in the medulla (Bofill et al., 1985) and an enlargement of the thymus (Berrih-Aknin et al., 1987). MG thymus displays all the components for the production of anti-AChR antibodies: AChR in TEC and myoid cells, B cells producing anti-AChR antibodies, and autoreactive anti-AChR T cells.

GC are structures usually found in secondary organs and are responsible for the proliferation, differentiation, and generation of memory B cells and plasma cells. B cell infiltration plays a central role in MG development, and the number of GCs is correlated with autoantibody titer (Berrih-Aknin et al., 1987). Patients with an HLA-DR3 haplotype are more prone to developing thymic follicular hyperplasia (Giraud et al., 2008).

Myoid cells could play a role in the establishment of MG because they expressed a functional AChR. This cell population is decreased and colocalized with GC in MG patients. Willcox et al. proposed that early anti-AChR antibodies present in the thymus target AChR in myoid cells, resulting in the activation of complement against those cells and the formation of GC (Willcox et al., 2008). Damage or destruction of those cells can lead to the release of AChR fragments and recapture by antigen-presenting cells, TEC or DC, potentially leading to sensitization against AChR.

In parallel with GC formation, neoangiogenesis processes have been observed in MG hyperplastic thymus. A high number of high endothelial vessels (HEV) is observed around GC and is correlated with the degree of hyperplasia. Lymphatic vessels (LEV) also develop in the MG thymus. HEV and lymphatic vessels favor the infiltration of peripheral cells in secondary lymphoid organs (SLO) and inflamed organs. The increase in HEV and lymphatic vessels indicates strong cell trafficking between the thymus and periphery.

This cellular trafficking is also tightly controlled by the abnormal expression of certain chemokines in the MG thymus. Overexpression of chemokines is responsible for the attraction and maturation of

lymphocytes and MG pathogenesis. HEV are implicated in the establishment of thymic hyperplasia by overexpressing the chemokine CXCL12/SDF-1 or CCL17, responsible for the homing of CXCR4 or CXCR7 and CCR4 positive cells, respectively (Cordiglieri et al., 2014; Weiss et al., 2013). Peripheral cells are coming into the thymus not only by blood vessels but also by lymphatic vessels. In the context of MG, new LEV overexpressed CCL21, a CCR7 ligand, leading to the recruitment of circulating peripheral cells such as naïve B cells (Berrih-Aknin et al., 2009).

Overexpression of CXCL13 by TEC has been found in MG patients and could participate in the infiltration of B cells in the thymus (Meraouna et al., 2006). Indeed, CXCL13 is a chemokine known to be implicated in the attraction of B cells and the formation of GC. Its receptor, CXCR5, is overexpressed on T follicular helper in GC, which are important cells for antibody production in SLO. TEC are also responsible for chemokine deregulation and overexpressed CXCL10 (Feferman et al., 2005). CXCL10 plays a role in the infiltration of T cells in inflamed sites. Its receptor, CXCR3, is also increased at the surface of T cells and leads to the recruitment of these cells in the thymus of MG patients.

Regarding these abnormalities, the thymus of MG patients could be considered tertiary lymphoid organs.

2.3.1.2.2. Thymoma

Thymoma is a tumor of the thymus with neoplastic epithelial cells. Thymomas are characterized by the nature of epithelial cells involved in the tumor: A = medullary thymoma, B1-2 = mainly or entirely cortical thymoma, AB = mix of medullary and cortical thymoma, and B3 = atypical thymoma (Suster and Moran, 2006). 90% of patients with thymoma develop an autoimmune disease, of which 30% is AChR-MG (Filosso et al., 2013). Thymoma associated with MG (MGT) is mostly types B1 and B2.

Among all MG patients, 10-20% have thymoma. MGT affects males and females equally, usually individuals above 50 years old. The thymus with thymoma has none or very few medullar compartments, which leads to an impaired negative selection of thymocytes. Thymoma is characterized by a deficit in AIRE (Ströbel et al., 2007) but not in the surrounding thymic tissue. This

deficiency could impair negative selection and explain the development of autoimmune diseases. Likely related to this deficiency, patients display lower Treg and lower FOXP3 expression in the thymus, affecting peripheral tolerance (Ströbel et al., 2004). Patients also have many different autoantibodies and 90% of patients express anti-titin and anti-ryanodine antibodies. Those two autoantibodies are used as markers for thymoma (Romi et al., 2005) and are usually found in patients with severe MG.

Patients can also have a thymoma not associated with MG. These patients are susceptible to developing MG if their thymus display GC and the overexpression of several cytokines in serum: IFN- γ , IL-1 β , and sCD40L (**ARTICLE 2 IN ANNEX: (Lefevre et al., 2020)**). These features could be used as biomarkers to predict the development of MG in patients with thymoma.

2.3.2. Immune imbalance in MG

T cell populations in MG

Several cell types from peripheral blood cells showed default in their functions. It has been demonstrated that conventional T cells and Treg are impaired in MG patients. Treg is a key cell for peripheral tolerance; two major subsets are thymic Treg and peripheral induced Treg. They regulate tolerance by their suppressive and anti-proliferating capacities over conventional T cells. However, in MG, though the number of Treg is normal, the suppressive function of Treg is severely defective, especially in the thymus (Truffault et al., 2020). There is a lower expression of Foxp3 in the Treg of MG patients, essential for their regulatory function (Balandina et al., 2005).

Conventional T cell proliferation is suppressed by Treg but in the MG context, they are resistant to Treg suppression (Gradolatto et al., 2014). The altered function of Treg and Tconv could be explained by an abnormal overexpression of pro-inflammatory cytokines. Indeed, pro-inflammatory IL-17 (Xie et al., 2016), IL-6, and IL-23 are overexpressed in EOMG patients and are already linked to several autoimmune diseases. In fact, IL-6 plays a dual role on T cells because it suppresses FoxP3 expression in Treg and is required for T cell helper 17 (Th17) differentiation (Yang et al., 2008). Th17 is a well-known pro-inflammatory T cell and the main cell expressing IL-17. An imbalance between Treg and

Th17 with a predominance for Th17 is found in MG patients. It has been shown that Treg can also express Th17 cell features by expression of IL-17, showing more of the T cell deregulation in MG (Gradolatto et al., 2012). IL-23, a key cytokine for the differentiation of T CD4⁺ cells toward Th17, is overexpressed by TEC, which could explain the T cell differentiation toward pathogenic Th17 (Villegas et al., 2019). Moreover, IL-23 contributes to the inflammatory environment and also to the formation of GC by attracting B cells and inducing antibody class switching (Peters et al., 2011).

Other T cell subtypes are implicated in the pathogenicity of the disease. T follicular helper (Tfh) cells, characterized by CD4⁺CXCR5⁺ expression, are usually found in GC and help B cells with affinity maturation and isotyping switching. It has been shown that this population is increased in peripheral blood and correlated with the severity of the disease. Moreover, the frequency of this population decreases after thymectomy, showing the importance of the thymus in the imbalance of this T cell population (Saito et al., 2005).

B cells in MG

Autoreactive anti-AChR B cells are found in the blood and lymph nodes of patients and can be associated with thymic hyperplasia and high serum antibody titers (Fujii et al., 1985). B cells from MG patients are hyperactivated, and isolated B cells from the hyperplastic thymus can spontaneously produce antibodies against AChR *in vitro* (Leprince et al., 1990). AChR patients exhibit a defect in central and peripheral B cell tolerance checkpoints (Lee et al., 2016). This could explain the expansion of autoreactive B cells.

A B cell subset called regulatory B cell (Breg) has an immunosuppressive function. It acts by producing the anti-inflammatory cytokine IL-10 and is in the blood and at the sites of inflammation. However, the Breg population in EOMG patients is decreased and presents a lower efficiency to produce IL-10 (Yilmaz et al., 2018).

Altogether, MG patients display altered peripheral tolerance by altered Treg and Breg but also an imbalanced toward pro-inflammatory T cells.

3. Implication of IFN-I in Myasthenia gravis

3.1. Link between IFN-I and MG thymus

3.1.1. IFN-I in early-onset MG patients

IFN-I can be linked to several autoimmune diseases, including MG. Several previous publications from the team showed IFN-I and IFN-III signatures in MG thymus (Le Panse et al., 2008) (Poëa-Guyon et al., 2005) with overexpression of IFN- β (Cufi et al., 2013). Actually, IFN- β is suspected to orchestrate thymic changes in EOMG patients. Indeed, IFN- β can induce the overexpression of α -AChR, the main target antigen in MG by TEC. Additionally, IFN- β increases slightly TEC apoptosis (Cufi et al., 2014a). In co-culture experiments pre-treatment of TEC with IFN- β increases the uptake of apoptotic TEC proteins by DC, which might lead to a cross-presentation of α -AChR by DC. This could explain the auto-sensitization against α -AChR and, therefore, autoantibody production.

IFN- β also induced overexpression of CXCL13 in TEC and CCL21 in LEV. This could favor the recruitment of peripheral cells and GC development in the thymus of patients. Moreover, IFN- β is implicated in the overexpression of BAFF in TEC (Cufi et al., 2014a). BAFF is a B cell pro-survival cytokine, and upregulation in the thymus of patients can promote survival of autoreactive B cells, which is the cell responsible for the pathogenicity of MG.

As previously described, the thymus of EOMG patients displays overexpression of IL-23, a major cytokine in the differentiation of T CD4⁺ cells toward pathogenic Th17. In fact, isolated TEC treated with an inducer of IFN-I overexpressed IL-23 (Villegas et al., 2019). Therefore, IFN- β not only acted on TEC but also sustained a pro-inflammatory thymic environment.

In conclusion, IFN-I plays a significant role in establishing thymic changes in the MG context.

IFN- β orchestrates thymic changes associated with myasthenia gravis

Endogenous ds nucleic acids

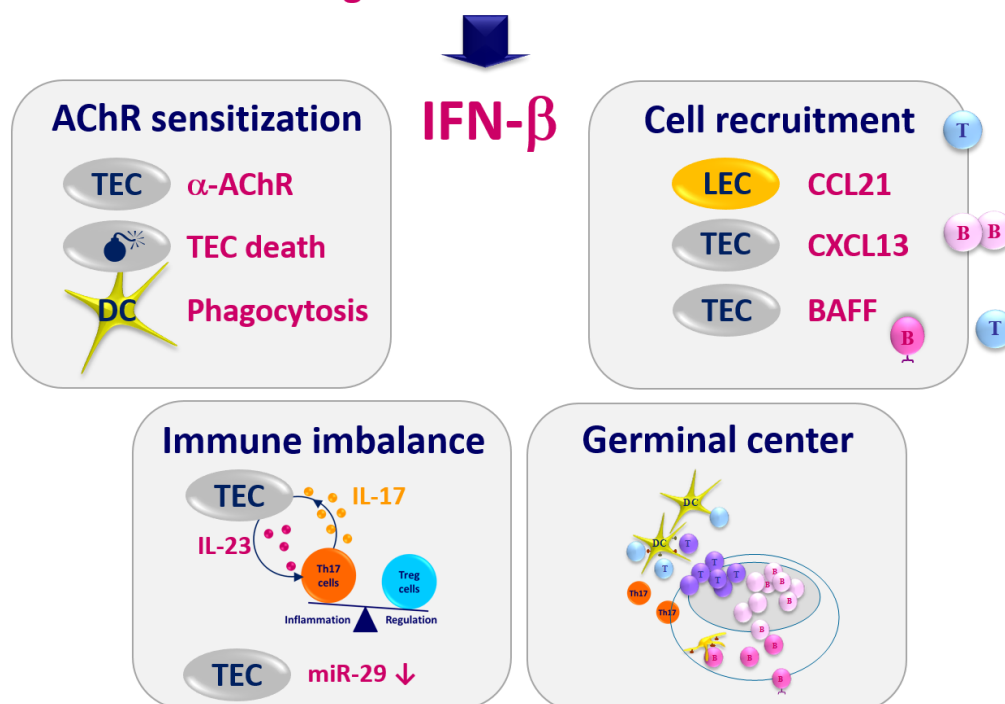


Figure 8: Orchestrator role of IFN- β in the thymic changes of MG

IFN- β leads to α -AChR sensitization by strongly inducing the expression of this antigen but also apoptosis in TEC. This could lead to cross-presentation by DC and induce the auto-sensitization against this antigen. In parallel, IFN- β favors T cell and B cell recruitment by inducing the expression of chemokines through stromal thymic cells but also favors an immune imbalance toward pathogenic Th17 and a decrease in miR-29. At the end, all these changes could induce the formation of GC and the production of anti- α -AChR antibodies.

Modified from (Robinet et al., 2017)

3.1.2. IFN-I in MG-associated thymoma

In MGT patients, autoantibodies against IFN-I are found in over half of MGT patients, especially against IFN- α 2, IFN- α 8, and IFN- ω but are rarer for IFN- β (Meager et al., 2003). Production of autoantibodies could be related to AIRE deficiency in MGT (Ströbel et al., 2007). Moreover, thymic extracted cells from the thymoma spontaneously express more anti-IFN- α 2 antibodies than the rest

of the non-thymomatous thymus (Shiono et al., 2003), which indicates plasma cell infiltration in the thymoma. The thymus is also associated with overexpression of TLR3, linked to the presence of the overexpression of IFN-I (Cufi et al., 2014b). It appears that IFN-I overexpression is dependent on the MG context and specific to the tumor environment because none of this expression is found in adjacent tissue.

Overexpression of the cytokines related to the autoantibodies IFN- α 2, IFN- α 8, IFN- ω , and IFN- β mRNA is found only in MGT patients and not in thymoma patients without MG. During my PhD, I participated to the characterization of the IFN-I signature in the periphery of MGT patients. Overexpression of IFN- α 2 and IFN- β seems to be confined to the thymus because no overexpression of these cytokines is found in the sera of patients (**ARTICLE 2 IN ANNEX: (Lefevre et al., 2020)**).

3.2. Origins of IFN-I in MG thymus

3.2.1. Implication of viral infection in IFN signature

The thymus is known to be a common target organ for viral infection (Savino, 2006). It is well documented that MG thymus overexpressed IFN-I (Cufi et al., 2013), an anti-viral molecule, leading to the possibility of the involvement of viral infection in the MG triggering factor.

The implication of viral infection in MG is greatly debated, particularly infection with the Epstein Barr virus (EBV). This virus targets B cells and is present in over 90% of the world's population. With this worldwide spread, it would be odd for EBV to be implicated in a rare autoimmune disease. However, the implication of EBV in several autoimmune diseases such as SLE, MS, and Rheumatoid arthritis (RA) has been demonstrated (Niller et al., 2008).

A study from Cavalcante et al. in 2010 showed that some MG patients are positive for EBV DNA intra-thymic but also positive for EBER protein, which is a latent protein of EBV, though healthy controls had none (Cavalcante et al., 2010a). Anti-type 1 nuclear antigen of Epstein-Barr virus antibody titer is not different between MG patients and healthy controls but EOMG cohorts have higher values (Csuka et al., 2012). The study conclude that EBV infection has a possible implication in the

establishment of MG. However, two other studies contradict this conclusion and did not find any EBV-positive patients (Kakalacheva et al., 2011; Meyer et al., 2011). For now, no definitive conclusion has been made. However, there are differences between those 3 studies, especially with regard to the treatment of the patients included (Serafini et al., 2011). Almost all patients included in the Cavalcante study were under immunosuppressive treatment, which is known to impair the immunosurveillance of EBV latent cells (Hislop et al., 2007), whereas the study of Kakalacheva included only patients without immunosuppressive treatments. No report of treatment was made in Meyer's study. These major differences can explain the divergence of results. A systematic review of the literature about the implication in EBV in MG (between 1980 and 2017) suggests that EBV plays only a minor role in the etiology of MG (Zhang et al., 2019).

The implication of viral infection in thymoma, particularly EBV, has also been tested. To date, several studies have been carried out but there is no conclusion. Cavalcante et al. showed that some MGT patients are EBV-positive but this seems to be linked to the MG and not specific to the thymoma. EBV-positive thymus might be a consequence of B cell EBV-positive infiltration rather than causing thymoma (Cavalcante et al., 2017).

Poliovirus has been found in macrophages of 4 of the 27 MG patients' thymuses, indicating the possibility of a viral contribution to MG (Cavalcante et al., 2010b).

In the search for a link between viral infection and MG, Dr. Rozen le Panse established a collaboration with Dr. Ian Lipkin (NY, USA) to investigate this hypothesis analyzing thymic samples with virochips (Wang et al., 2002). However, no specific viral signature has been found in the thymus of MG patients (unpublished data).

To develop the hypothesis of viral infection, Cufi et al. investigated the possible infection of human papillomavirus (HPV) in MGT patients. In fact, MGT patients overexpressed the tumor suppressor p16, a potential marker for HPV infection. However, the study showed no evidence of HPV infection in patients (Cufi et al., 2014b).

Altogether, the results of several studies did not link IFN-I expression and potential viral infection in MG.

3.2.2. Activation of dsRNA innate signaling pathways

The study of human samples for viral implication in MG is inconclusive so far. One reason could be the timing of the study, i.e., too far from the disease onset. So, experimental approaches have been carried out. To test this hypothesis *in vitro*, TEC have been treated with different TLR agonists to investigate whether it could trigger IFN-I expression. Cufi et al. demonstrated that only the agonist of TLR3, Poly(I:C) molecule mimicking dsRNA from a viral infection, greatly induces IFN-I but also α -AChR in TEC (Cufi et al., 2013). Furthermore, this study demonstrated that the expression of α -AChR by TEC is induced by IFN-I, and particularly IFN- β . IFN- β has a pleiotropic effect on thymic cells and especially the induction of CXCL13, CCL21, and BAFF, which are cytokines responsible for B cell infiltration and survival. All those important cytokines are overexpressed in MG thymus. Interestingly, mice treated with Poly(I:C) for one week present an overexpression of IFN- β in the thymus but also an increase of α -AChR. Moreover, IFNAR KO mice treated with Poly(I:C) do not overexpress α -AChR in the thymus, which means that overexpression of α -AChR is due to IFN-I signaling. The thymuses of mice treated with Poly(I:C) also display overexpression of CXCL13 and CCL21, like human MG thymus. An increase in B cell infiltration is found in the thymuses of Poly(I:C) treated mice, surely due to the expression of chemokines (Weiss et al., 2016). After 6 weeks of Poly(I:C) injection, anti-AChR antibodies are found in the sera of mice but there are no more thymic changes. Long-term treatments lead to the development of MG in mice characterized by muscular weakness and a decrease of α -AChR in diaphragm muscle (Cufi et al., 2013). In summary, experimental approaches to investigate the role of IFN-I in MG etiology concord with what we found in MG patients and prove the important role of IFN-I.

3.2.3. Altered miRNA expression

miRNA are potent modulators of protein expression and are, therefore, involved in many physiological and pathophysiological processes. Specific miRNA are known to be involved in thymic pathogenesis associated with MG (Cron et al., 2018; Sengupta et al., 2018). miR-146, involved in retro-control of IFN-I, is downregulated in the thymus of MG patients and this deficiency is may contribute to persistent innate immune activation and inflammation (Bortone et al., 2020). By contrast, miR-155 is upregulated in MG patients and could promotes type I IFN signaling by targeting SOCS1 (Pathak et al., 2015; Wang et al., 2014). Papadopoulou et al. demonstrated that miRNA are essential to protecting thymic architecture. Using conditional knock-out mice for Dicer in TEC (Foxn1Cre Dicerfl/fl), they observed a premature involution and the appearance of epithelial voids with dense foci of B cells. Also, Dicer-deficient mice are hypersensitive to Poly(I:C) in line with the increased expression of Ifnar1 in TEC. This latter effect is mediated by the miR-29a/b-1 cluster, as miR-29a targets Ifnar1 and increases sensitivity to Poly(I:C) due to an increased expression of Ifnar1 in TEC (Papadopoulou et al., 2012).

These observations suggest a link between miR-29a and the IFN-I signature observed in the MG thymus. We investigated the implication of DICER and the miR-29 family in thymic changes in early-onset MG. In the human thymus, decreased expression of DICER and miR-29a-3p was observed in MG patients and also in TEC upon IFN- β treatment. Yet, it is not clear whether the decreased expression of miR-29 subtypes in MG is either a consequence or a causative factor of the thymic inflammation. In any event, our results indicated that a reduction in miR-29 subtypes may contribute to the pathophysiological process involved in MG by favoring the emergence of pro-inflammatory Th17 cells.

4. Objective

MG is an autoimmune disease with autoantibodies against different proteins of the NJM. EOMG patients are characterized by autoantibodies against AChR and the involvement of the thymus in the pathology (Le Panse et al., 2010). It has been proven in many ways that IFN-I, in particular IFN- β , plays a central role in the establishment of thymic changes in MG. IFN- β induces cytokine and chemokine expression, leading to B cell infiltration and auto-sensitization against α -AChR. This thymic IFN-I signature is observed even long after disease onset and it is unclear what causes this chronic production of IFN- β . The team in which I did my PhD is investigating this IFN-I signature using different approaches. In this context, I was involved in a study on mirRNAs to analyze the implication of miR-29 in the excessive thymic IFN-I signalization. We observed a decreased expression of miR-29 in the thymus of MG patients but showed that it was, in fact, a consequence of IFN- β overexpression. Nevertheless, this study demonstrated that a thymic decrease in miR-29 expression could favor the emergence of inflammatory Th17 cells that are known to be involved in MG pathophysiology (**ARTICLE 3 IN ANNEX: (Cron et al., 2020)**).

In the past few years, Mendelian and autoimmune diseases have been related to an abnormal chronic expression of IFN-I leading to autoinflammation (Crow et al., 2019). They are part of the type-I interferonopathy family. The major aim of my PhD project was to investigate whether the early-onset form of AChR MG could be related to this interferonopathy family.

First, I studied the IFN-I signature in the periphery as observed in interferonopathies (Rodero and Crow, 2016). I analyzed serum levels of IFN-I subtypes and searched for an IFN-I in PBMC of patients, whether or not they were thymectomized. In parallel, I was involved in another project investigating the serum levels of IFN-I subtypes in thymoma-associated MG (**ARTICLE 2 IN ANNEX: (Lefevre et al., 2020)**).

Next, the main part of my thesis was to understand the origin of the IFN-I signature in the thymus of EOMG patients. Based on other diseases associated with sterile inflammation and IFN-I production,

we suspected the implication of endogenous nucleic acids in the thymic IFN-I signature. I studied the effects of molecules mimicking endogenous nucleic acids on IFN-I and α -AChR expression in human TEC *in vitro* and in the thymus of mice *in vivo*. I then suspected that endogenous nucleic acids were released by apoptotic/necrotic thymocytes in the thymus. To assess this hypothesis, I measured the expression of IFN-I and α -AChR in TEC co-cultured with necrotic thymocytes but also in the thymus of mice injected with dexamethasone, a potent inducer of thymocyte apoptosis. Finally, I wanted to understand what could induce the increase in necrotic thymocytes that were susceptible to release their nucleic acids and induce thymic inflammation. An abnormal clearance of apoptotic cells leads to secondary necrosis and the release of cell content. Macrophages, cells implicated in the clearance of apoptotic cells, were quantified in the thymus of MG patients and healthy donors. To understand the implication of macrophages in IFN-I production, I used a mouse model with an inducible deficiency of macrophages. Thymic changes such as apoptosis or necrosis rate were measured as well as IFN-I and α -AChR expression. The results obtained are fully described in the main manuscript of my thesis (**ARTICLE 1; Payet et al. to be submitted**).

Results

Article 1

Endogenous nucleic acids induce an organ-specific interferon type I signature in Myasthenia gravis thymus

Myasthenia gravis (MG) is an autoimmune disease mediated by autoantibodies against the acetylcholine receptor (mainly α -AChR). The thymus plays a primary role in early-onset MG and is characterized by chronic overexpression of interferon (IFN)- β . Type-I interferonopathy family diseases display a chronic IFN-I expression in the periphery. We first studied the IFN-I signature in the periphery of MG patients. Serum levels of IFN- β and IFN- α were investigated and IFN-I, receptors, and IFN-induced genes were analyzed in PBMCs. No IFN-I signature was observed in the periphery in early-onset MG. Therefore, we concluded that MG might be only an organ-specific interferonopathy. Next, we suspected that endogenous nucleic acids could be responsible for IFN-I expression in MG. Focusing on the thymic overexpression of IFN- β in MG, I demonstrated that a molecule mimicking endogenous nucleic acids (Poly(dA:dT)) or a molecule part of IFN-I signaling (2'3'-cGAMP) induced the over-expression of IFN- β and the subsequent expression of α -AChR in human TEC or in the thymus of mice. Endogenous nucleic acids were suspected to be released by necrotic cells. To investigate this hypothesis, I induced strong thymocyte necrosis *in vitro* or *in vivo* using dexamethasone. I showed an increased IFN-I signature and the expression of α -AChR in TEC and in the thymus of treated mice. Investigating the cause of thymocytes necrosis, I showed in early-onset MG a significant decrease in thymic macrophages, i.e., cells responsible for the clearance of apoptotic cells. In mice, depletion of thymic macrophages led to an increase in necrotic thymocytes associated with an IFN-I signature and α -AChR expression. These results suggest that a decrease in the number of macrophages in early-onset MG thymus may alter the processing of apoptotic thymocytes upon acute stress. This would lead to the release of endogenous nucleic acids from necrotic thymocytes and the activation of innate signaling pathways. Upon contact, with endogenous nucleic acids, TEC would overproduce IFN- β , which induces α -AChR. In this inflammatory context, this would lead to the self-sensitization against α -AChR and thymic changes leading to MG.

Endogenous nucleic acids induce an organ-specific interferon type I signature in Myasthenia Gravis thymus

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Abbreviations: AChR: Acetylcholine Receptor, IFN: Interferon, IFN- α/β receptor: IFNAR, NA: Nucleic Acid, EOMG: Early-Onset Myasthenia Gravis, MG: Myasthenia Gravis, PBMC: Peripheral Mononuclear Blood Cells, TEC: Thymic Epithelial Cell

Abstract

Objective

Myasthenia gravis (MG) is an autoimmune disease mediated by antibodies against the acetylcholine receptor (mainly α -AChR). The thymus plays a primary role in early-onset MG and is characterized by chronic overexpression of interferon (IFN)- β . We thoroughly studied the IFN-I signature in the periphery and thymus of MG patients. Next, we further analyzed the contribution of endogenous nucleic acids in the pathogenesis of MG.

Methods

Serum levels of IFN- β and IFN- α were investigated by ELISA and Simoa, respectively. IFN-I subtypes, receptors, and IFN-induced genes were analyzed in PBMCs by RT-PCR. Thymic epithelial cells (TECs) were cultured with synthetic nucleic acids (Poly(dA:dT), HSV-60, CpG), 2'3'-cGAMP, or necrotic thymocytes. *In vivo*, mice were treated with dexamethasone to induce thymocyte apoptosis/necrosis, and the thymus were analyzed. Thymic sections from control and MG donors were labeled to analyze CD68⁺ macrophages, and the impact on the thymus of a decreased number of macrophages induced by prolonged treatment with an anti-CSF1R antibody was analyzed in mice.

Results

No IFN-I signature was observed in the periphery in early-onset MG. Focusing on the thymic overexpression of IFN- β in MG, we demonstrated that Poly(dA:dT), 2'3'-cGAMP, and apoptotic/necrotic thymocytes induced the over-expression of IFN- β and the subsequent expression of α -AChR by TECs. In mice, dexamethasone induced strong necrosis of thymocytes associated with a thymic IFN-I signature and an increased expression of α -AChR. A significant decrease in thymic macrophages was shown in early-onset MG. In mice, depletion of thymic macrophages led to an increase in necrotic thymocytes associated with an IFN-I signature and α -AChR expression.

Interpretations

These results suggest that a decrease in the number of macrophages in early-onset MG thymus may alter the processing of apoptotic thymocytes upon acute stress. This would lead to the release of endogenous nucleic acids from necrotic thymocytes and the activation of innate signaling pathways. Once in contact with endogenous nucleic acids, TECs would overproduce IFN- β , which induces α -AChR. In this inflammatory context, this would lead to self-sensitization against α -AChR and thymic changes leading to MG.

Introduction

Acquired Myasthenia Gravis (MG) is a neuromuscular disease characterized by invalidating muscle weaknesses. MG is a prototypical organ-specific autoimmune disease with a well-defined antigenic target, as autoantibodies are directed mainly against the acetylcholine receptor (AChR), in particular, the α -AChR subunit ¹. In early-onset AChR MG (EOMG), the thymus is the effector organ and its removal alleviates the symptoms of the disease ². The MG thymus displays functional and morphological abnormalities characterized by abnormal B cell infiltration leading to ectopic germinal center development (thymic follicular hyperplasia). Moreover, the MG thymus includes all the components of the anti-AChR response: the expression of AChR subunits by thymic epithelial cells (TECs) in the context of central tolerance ³ and myoid cells ⁴, the presence of B cells producing anti-AChR antibodies, ^{5 6} and the presence of anti-AChR autoreactive T cells ⁷.

In MG, an interferon (IFN)-I signature is detected in the thymus. This is especially linked to the overexpression of IFN- β ⁸ that seems to be the main orchestrator of thymic changes observed in EOMG patients: 1) IFN- β favors the selective sensitization against AChR by specifically inducing α -AChR expression in TECs, and increasing TEC death and the uptake of TEC proteins by dendritic cells ⁹. 2) IFN- β increases thymic expression of CXCL13 and CCL21, two chemokines involved in germinal center development and overexpressed in the EOMG thymus. 3) IFN- β induces the overexpression of the B cell activating factor (BAFF), which favors autoreactive B cell survival. 4) IFN- β stimulates the development of pathogenic Th17 cells in the thymus (**ARTICLE 3 IN ANNEX:** ¹⁰¹¹) Also, a causative role of IFN-I is supported by a mouse model where injections of Poly(I:C) induce thymic changes that lead to an anti- α -AChR response ⁸⁻⁹.

The discovery of the etiological mechanisms involved in MG lies in the understanding of signals that drive this abnormal thymic IFN-I signature. The thymus is a common target organ for infection diseases ¹² and IFN-I subtypes are transiently produced in response to viral infections. However, the IFN-I signature is detected in the MG thymus, even long after disease onset ⁸. In 2011, Crow et al. defined a new group of Mendelian disorders characterized by the chronic overexpression of IFN-I ¹³.

Mutations affect genes involved in nucleic acid (NA) processing and recognition, or genes involved in the negative retro-control of IFN-I signalization¹⁴. Some autoimmune diseases tend to share features with Mendelian interferonopathies with an IFN-I signature in peripheral blood and target tissues, such as in systemic lupus erythematosus, dermatomyositis, and Sjögren's syndrome¹⁵⁻¹⁷. Plasmacytoid dendritic cells are the main IFN-I producers upon viral infection but non-hematopoietic cells are also responsible for IFN-I production in the context of autoimmunity¹⁸.

We then investigated whether EOMG shared characteristics with interferonopathies, doing so by analyzing the IFN-I signature in the blood and thymus of MG patients. We showed that MG is a thymus-specific acquired interferonopathy. Using *in vitro* and *in vivo* approaches, we demonstrated that endogenous NAs released by necrotic thymocytes activated IFN-I release and α -AChR expression by TECs. In the context of MG, we observed a decreased number of thymic macrophages. This could alter the proper phagocytosis (efferocytosis) of apoptotic thymocytes, and necrotic thymocytes would release their intracellular content. Finally, endogenous NAs would activate innate immunity pathways and trigger sterile inflammation at the basis of thymic changes leading to the EOMG.

Methods

Human blood and thymic samples

Control serum from male and female donors (n=14, 23-50 years old) and PBMC (n=11, 25-52 years old) were obtained from the French blood establishment (EFS). MG patient samples were from age-/sex-matched donors for serum (n=44, 12-49 years old) and PBMC (n=24, 20-57 years old). MG patients were treated only with cholinesterase inhibitors and not with immunosuppressive therapies. However, 16 patients were thymectomized.

For immunohistochemistry, thymic biopsies were obtained from the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France), where early-onset AChR-positive MG patients (n=9, 8-43 years old) underwent thymectomy, and from age-/sex-matched non-MG controls (n=5, 6-44 years old) from cardiovascular surgery. TECs were prepared from infant thymus obtained from cardiovascular surgery.

No MG patients displayed thymoma or infectious diseases. Studies on blood and thymic samples were approved by local ethics committees (RCB 2006-A00164-47 and RCB 2010-A00250-39).

Serum cytokine measurement

IFN- α serum levels from controls and MG patients were measured using a Quanterix Simoa assay developed by Dr. Darragh Duffy's team as previously described. Samples were diluted to half, and this assay allowed for the detection of all IFN- α isoforms. IFN- β serum levels were assessed by an ELISA (PBL-41415, Enzo Life Science, Villeurbanne, France) on serum samples diluted to half.

TEC cultures

TECs were obtained from thymic explant cultures as previously described⁸. After one week, TECs were collected and seeded overnight at $1.4 \cdot 10^5$ cells/cm² in RPMI-5% horse serum. TECs were then cultured in RPMI-0.5% horse serum and treated for 24h with either Poly(I:C) 200 μ g/mL (tlrl-pic), Poly(dA:dT) 100 μ g/mL (tlrl-patn), CpG 5 μ g/mL (tlrl-2216), 2'3'-cGAMP 100 μ g/mL (tlrl-nacga23), or HSV-60 100 μ g/mL (tlrl-hsv60n). All products were purchased from Invivogen (Toulouse, France).

Interferon- α/β receptor subunit 2 (IFNAR2) blocking antibody 10 μ g/mL (PBL-21385-1, Enzo Life Science) was added 1h before the other reagents.

For co-culture experiments, TECs were prepared as above and co-cultured with thymocytes induced in apoptosis. The induction was done by incubating thymocytes for 4h with 50 μ M dexamethasone (D4902, Sigma-Aldrich, St. Quentin Fallavier, France). Thymocytes were washed to remove dexamethasone and seeded with TECs for 24h (Thymocyte/TEC ratio=20:1). Before TECs were collected, the co-culture was thoroughly washed by D-PBS to get rid of the thymocytes.

RT-qPCR

Total RNA from human TECs or mouse thymic biopsies was extracted in Trizol (15596018, Life Technologies, Villebon-sur-Yvette). 500ng of RNA was retro-transcribed using AMV (10109118001, Roche Life Science, Meylan, France) for 1h at 42°C with oligo-dT (18418012, Life Technologies). Quantitative PCR reactions were performed with the LightCycler 480 SYBR Green Master Mix on the LightCycler® 480 System (4887352001, Roche Life Science). Primer sequences are listed in table 1. Relative mRNA expressions were normalized with GAPDH or 28S.

Immunohistochemistry

Cryosections of thymic samples (7 μ m) were fixed in ice-cold acetone for 20 minutes and unspecific binding sites were blocked with 5% BSA for 20 minutes. Sections were stained overnight at 4°C with antibodies against keratin 5 (905501, Biolegend, Amsterdam, Netherlands) for medullary TECs, CD68 (MAB20401, R&D systems, Noyal Châtillon sur Seiche, France), and CD163 (556017, Becton Dickinson, Le Pont de Claix, France) for macrophages or CD123 (555642, Becton Dickinson) for plasmacytoid dendritic cells and CD208 (IM3448, Beckman Coulter, Villepinte, France) for DC-LAMP. Next, sections were stained with fluorescent secondary antibodies: Donkey anti-mouse alexa 594 (A-21203, Life Technologies) or Chicken anti-rabbit alexa 488 (A-21441, Life Technologies) for 1h30 at room temperature. Images were acquired with a ZeissAxio Observer R1 Inverted Microscope. The

number of macrophages or dendritic cells was counted in 5 mosaic pictures containing 25 fields of each thymic section.

Animals

4-5 weeks old C57/bl6J mice (Janvier Labs, Saint Berthevin, France) were treated by intraperitoneal injections of either Poly(I:C) 200µg/injection, dexamethasone 5mg/kg/injection, Poly(dA:dT) 100µg/injection, 2'3'-cGAMP 25µg/injection, or saline solution 3 times per week.

Mice were treated with an anti-CSF1R antibody 300µg/injection (NBP2-43363, BioTechne, Noyal Châtillon sur Seiche, France) every 3 days for 4 weeks.

Mice were sacrificed after one week and the thymus was cut in two: One part proceeded freshly for flow cytometry analyses and the other part was stored at -80°C.

Flow cytometry

Thymuses from mice were crushed and filtered through 60µm nylon. Thymocytes were labeled with Annexin V (556419, Becton Dickinson) and propidium iodine (PI) (51-66211E, Becton Dickinson) in Annexin V binding buffer for 15 minutes. Cells were monitored using the Beckman Coulter Cytoflex S apparatus.

Statistical analysis

For 2-by-2 comparisons, the non-parametric Mann-Whitney test was applied. For multiple comparisons, a one-way ANOVA test was applied with Dunn's post hoc tests. Correlation analyses were performed using Spearman's correlation coefficient for non-Gaussian distributed variables, with $p < 0.05$ considered significant.

Results

Search for an IFN-I signature in the periphery

First, we investigated the serum levels of IFN- β (Fig 1A) and IFN- α isoforms (Fig 1B) by ELISA and Simoa, respectively. No differences were observed between controls and MG patients. Furthermore, among MG patients, no differences were observed between patients thymectomized or not (Figs 1A-B).

We also measured mRNA expression in PBMCs from controls and MG patients for IFN- β (Fig 1C), IFN- $\alpha 2$ (Fig 1D), and their receptor subunits IFNAR1 (Fig 1E) and IFNAR2 (Fig 1F). Although a slight increasing trend was observed for IFN- β mRNA for MG patients (Fig 1C), no significant differences were detected for these four genes in MG patients whether or not they were thymectomized. The assessment of an IFN-I signature is usually carried out by measuring ISG induction, classically OAS1 and MX1 but also six ISGs identified by Dr. Crow to define interferonopathies¹⁹. We measured the mRNA expression of these ISGs in PBMCs from MG patients. No significant differences were observed (Figs 1 I-N) in MG patients (thymectomized or not) as compared to controls, except for RSAD2 (Fig 1N).

Overall, these results indicate that no IFN-I signature was in the periphery of MG patients, as measured in serum and PBMCs.

Investigation of the origin of the IFN- β signature in the thymus

We previously demonstrated that Poly(I:C), a double-stranded(ds)RNA mimicking both viral or endogenous dsRNA, strongly induces in TECs the expression of IFN- β and α -AChR, the main antigen in MG⁸. Here, we investigated the *in vitro* effect on TECs of molecules mimicking dsDNA of bacterial (unmethylated CpG) or viral (HSV-60, a synthetic herpes simplex viral DNA) origin, endogenous dsDNA (Poly(dA:dT)), and a molecule involved in intracellular signalization of dsDNA (2'3'-cGAMP). The overexpression of IFN- β (Fig 2A) and α -AChR (Fig 2B) mRNA was observed in TECs treated for 24h with Poly(dA:dT) and 2'3'-cGAMP but not with molecules mimicking pathogen infections. Although

Poly(dA:dT) and 2'3'-cGAMP induced IFN- β and α -AChR in TECs, they were not as potent as Poly(I:C) (Fig S1A-B). Of note, other IFN-I subtypes, such as IFN- α 2 and IFN- α 8, were also induced by Poly(dA:dT), 2'3'-cGAMP, and Poly(I:C) (Fig S1 C-D). The induction of α -AChR expression upon stimulation with Poly(dA:dT) or 2'3'-cGAMP was specific, as no inductions were observed for β -AChR (Fig S2A) or other tissue-specific antigens such as those expressed by TECs (Fig S2A).

We also analyzed in kinetic the expression of IFN- β and α -AChR mRNA upon the activation of the dsDNA signalization with 2'3'-cGAMP. We observed that IFN- β mRNA expression peaked at 3h and decreased afterward (Fig 2C), while α -AChR expression peaked at 24h (Fig 2D). This shift suggests that α -AChR expression could be mediated in an autocrine manner by the expression of IFN- β by TECs. To verify this hypothesis, TECs were pre-treated with an IFNAR blocking antibody before being stimulated with either 2'3'-cGAMP (Fig 2E) or Poly(dA:dT) (Fig 2F) for 24h. The results showed a significant decrease in the expression of α -AChR mRNA when IFN-I signalization was blocked.

Experiments have been done in which mice were injected with 2'3'-cGAMP or Poly(dA:dT). *In vivo*, we observed a significant increase in IFN- β and IFN- α 2 mRNA in the thymus, associated with a specific increase in α -AChR mRNA (Figs S3A-J).

Altogether, these results suggested a specific implication of endogenous nucleic acids, dsDNA and dsRNA, in the expression by TECs of IFN- β that further induced that of α -AChR.

Analysis of the effects of necrotic thymocytes on IFN- β and α -AChR expression by TECs

Endogenous nucleic acids are known to be released in the cellular environment by necrotic cells that lose the integrity of their plasma and nuclear membranes. We investigated the impact of co-culturing necrotic cells with TECs. Dexamethasone is a potent inducer of apoptosis for thymocytes²⁰. Here, thymocytes were induced in apoptosis/necrosis by a 4h pretreatment with dexamethasone that increased the percentage of annexin V⁺/PI⁺ (Figs 3A-B). Thymocytes were then washed to get rid of dexamethasone and co-cultured for 24h with TECs. We observed that apoptotic/necrotic thymocytes increased the expression of IFN- β mRNA at 24h as compared to co-cultures with untreated thymocytes (Fig 3C). We next showed clearly that apoptotic/necrotic thymocytes increased the

expression of α -AChR mRNA at 24h by TECs (Fig 3D). The effect of apoptotic/necrotic thymocytes was specific to α -AChR and not observed for β -AChR (Fig 3E) or ε -AChR (Fig 3F). Furthermore, using only the supernatant of apoptotic/necrotic thymocytes on TECs, an induction of α -AChR mRNA at 24h by TECs was also observed (data not shown).

These data suggest that the release of the intracellular content of necrotic thymocytes, containing nucleic acids, could induce the expression by TECs of IFN- β and, subsequently, that of α -AChR.

Analysis of the in vivo effects of necrotic thymocytes on IFN-I subtypes and α -AChR expression in the thymus

Dexamethasone is a potent inducer of acute thymic involution due to thymocytes apoptosis²¹. We then investigated the impact of thymocyte apoptosis on the thymus of mice. Seven days after dexamethasone treatment, thymus weight was significantly decreased in treated mice (Fig 4A) but not the total weight of the mice (data not shown). A strong decrease in the total number of thymocytes was observed (Fig 4B) but the thymic stroma was not affected, as no change in keratin 5 mRNA expression was detected (Fig 4C). Thymic involution was due to the strong induction in thymocyte apoptosis (Fig 4D) and necrosis (Fig 4E) in injected mice.

In parallel, we observed an increased expression of IFN-I subtypes, IFN- β (Fig 4F), and IFN- α 2 (Fig 4G) mRNA and also of α -AChR mRNA (Fig 4H). No increased expression was measured for β -AChR mRNA (Fig 4I). Furthermore, a strong correlation between IFN- β or IFN- α 2 and α -AChR mRNA expression in the thymus of mice injected with dexamethasone was observed (Figs 4J-K).

All these results suggest that necrotic thymocytes induced upon dexamethasone treatment could trigger the expression of IFN-I leading to α -AChR expression in the thymus.

Investigation of thymic macrophages in the pathology

Apoptotic cells are normally efferocytozed by phagocytic cells. This prevents apoptotic cells from entering secondary necrosis, leading to leakage of their cellular content. Macrophages play a major role in the efferocytosis of apoptotic thymocytes^{22 23}. By immunofluorescence, we detected a

significant decrease in the number of CD68⁺ macrophages in the thymus of MG patients as compared to controls. This decrease was observed in both cortical and medullary regions (Figs 5A-B).

To determine if a decrease in thymic macrophages could be related to the IFN-I signature observed in the MG thymus, and the abnormal increased expression of α -AChR, the autoantigen involved in MG, we used a mouse model. Animals were injected with an anti-CSF1R antibody for 4 weeks. CSF1 is a molecule implicated in the renewal and differentiation of tissue macrophages, and prolonged blocking of its receptor induces macrophage depletion²⁴. We observed a non-significant decrease in thymus weight that we did not explain (Fig 5C), as no change in the total number of thymocytes (Fig 5D), nor in Keratin 5 mRNA expression (Fig 5E), was observed. We detected a slight increased proportion of necrotic thymocytes (Fig 5F), associated with a tendency toward less apoptotic thymocytes (Fig 5G). Although the increase in necrotic thymocytes was light, we measured higher thymic levels of IFN- α 2 mRNA and a tendency for IFN- β mRNA, together with an increase in α -AChR mRNA (Fig 5H) but not for β -AChR (Fig 5I).

These results suggest that a default in thymic macrophages could lead to thymic inflammation as observed in MG patients.

Discussion

The thymus of EOMG patients is characterized by a long-lasting IFN-I signature, associated mainly with the high expression of IFN- β ⁸. We wondered whether a peripheral IFN-I signature could also be detected in EOMG patients. However, no significant increased serum levels for IFN- α isoforms or IFN- β were detected, and clearly no IFN-I signature was observed in PBMCs from EOMG patients. In a whole-transcriptome sequencing study on PBMCs from EOMG patients, Barzago et al. detected enriched functional pathways related to “infectious disease” and “inflammatory response-associated” categories. However, a detailed analysis of dysregulated genes revealed no obvious IFN-I signature²⁵. In both our study and that of Barzago et al., no changes were detected for thymectomized or non-thymectomized EOMG patients, indicating that the chronic IFN-I signature is contained to the thymus. In contrast to EOMG, chronic peripheral activation of the IFN-I pathway seems to be linked to autoimmune diseases with systemic involvement and characterized by the presence of anti-nuclear antibodies such as systemic lupus erythematosus, dermatomyositis and Sjögren’s syndrome,^{15–17,26}. In these autoimmune diseases, an IFN-I signature is also detected in specific organs, such as in the salivary glands for Sjögren’s syndrome¹⁶ and the muscle and skin for dermatomyositis¹⁵. MG is a prototypical organ-specific autoimmune disease for which the target organ is the neuromuscular junction on skeletal muscle. Of note, the muscle is not inflammatory and no IFN-I signature has ever been observed in MG muscle²⁷. In EOMG, the IFN-I signature is detected only in the thymus. An IFN-I signature is also observed in the thymus of thymoma-associated MG²⁸ without peripheral signature²⁹. Altogether, this leads us to consider that MG is a thymus-specific kind of acquired interferonopathy.

As the thymus is thought to initiate the anti-AChR response associated with EOMG, a key issue is the understanding of signals that drive the chronic expression of IFN-I. Our team previously demonstrated that Poly(I:C), a dsRNA molecule, induces the overexpression of IFN- β by TECs that, in turn, leads to the specific expression of α -AChR⁸. Here, we demonstrated that Poly(dA:dT) and 2’3’-cGAMP induced the expression of IFN-I subtypes, in particular IFN- β , and of α -AChR. The expression

of IFN- β peaked at 3h and that of α -AChR was delayed. Blocking the IFN-I receptor strongly inhibited α -AChR expression. These results suggest that dsDNA signalization via STING could induce the IFN-I release by TECs and also the subsequent specific overexpression of α -AChR. Similarly, *in vivo*, Poly(dA:dT) and 2'3'-cGAMP injections to mice increased the IFN-I thymic signature and α -AChR expression, as observed with Poly(I:C) injections ⁸.

We did not observe any effect using HSV-60 or CpG, synthetic molecules mimicking dsDNA from viral or bacterial origin, respectively. Poly(I:C) is usually considered a molecule mimicking viral infection but it can also mimic dsRNA from an endogenous origin ³⁰. This suggests that endogenous double-stranded NAs could be responsible for the IFN-I signature observed in the MG thymus. The fact that endogenous dsRNA and dsDNA use innate signaling pathways ³¹ may explain the long-standing hypothesis that MG may be due to pathogen infection. Indeed, various pathogens have been associated with MG but no clear evidence supports a causal link between a specific pathogen and the onset of MG ³².

Inappropriate activation of innate signaling pathways by endogenous NAs could be due to the release of the intracellular content of necrotic cells. Indeed, we demonstrated that the co-culture of apoptotic/necrotic thymocytes with TECs induced IFN- β and α -AChR expression, similar to what we observed with synthetic NAs. These *in vitro* observations were reproduced *in vivo* by inducing thymic acute stress. Dexamethasone injections are known to lead to thymic involution due to the huge induction of thymocyte death ²⁰. We showed that the increase in the number of apoptotic/necrotic thymocytes led to the expression of IFN-I subtypes, such as IFN- β and IFN- α 2, and of α -AChR. As for dexamethasone, the effects of Poly(I:C) injections described by Cufi et al. could also be due to the acute thymic involution induced by this molecule ⁸. However, we cannot exclude a direct effect of this dsRNA molecule. Indeed, Poly(dA:dT) and 2'3'-cGAMP injections were also able to induce IFN- β and α -AChR expression without thymic involution, and Demoulins et al. observed increased levels of IFN-I subtypes as soon as 1h after Poly(I:C) injection ³³. The implication of endogenous NAs in inflammatory disorders and autoimmunity is increasingly suspected. For example, defective

clearance of endogenous NAs from dying cells is implicated in the initiation of systemic lupus erythematosus³⁴. Endogenous NAs also induce IFN-associated responses in keratinocytes and are involved in the pathogenesis of cutaneous lupus erythematosus³⁵.

Clearance of cellular debris is clearly required to maintain tissue homeostasis. Under physiological conditions, apoptotic cells are taken over by phagocytes, such as macrophages, through a process called efferocytosis. In contrast to phagocytosis, efferocytosis is immunological silent to avoid the induction of a sterile inflammation. If apoptotic cells are not eliminated, they progress to a secondary necrosis stage and locally release their intracellular content, such as endogenous NAs. We clearly observed a decreased number of macrophages in the thymus of MG patients. In parallel, we observed that a decreased number of thymic macrophages in mice was associated with an IFN-I signature and the induced expression of α -AChR.

The thymus is essential to shaping the T cell repertoire to optimize the immune response and avoid autoimmunity³⁶. The thymus is a site of intense cell death in the context of positive and negative selection during which about 95% of the thymocytes are eliminated³⁷. In addition, thymocyte apoptosis is strongly induced upon acute stress such as a viral infection, stress, or pregnancy³⁸. It is thus evident that efferocytosis of apoptotic thymocytes is a critical physiological process to maintain thymic homeostasis. We hypothesized that a default in thymic macrophage number and/or function could alter apoptotic thymocyte efferocytosis. Necrotic thymocytes would thus release an abnormal amount of endogenous NAs, provoking the inflammatory response observed in the thymus of EOMG. Nevertheless, the thymus is known to have the ability to rapidly recover upon acute stress³⁹. Consequently, we suspect that an abnormal resolution of inflammation is also implicated in MG pathogenesis.

We previously showed that IFN- β plays a central role in thymic events leading to MG by triggering the overexpression of α -AChR, likely leading to thymic dendritic cell sensitization against this autoantigen, the abnormal recruitment of peripheral cells and germinal center formation, the differentiation of pathogenic Th17 cells, and the survival of autoreactive B cells⁹⁻¹¹. Here, we

demonstrated that the thymic IFN-I signature could be elicited by the release of endogenous NAs from necrotic thymocytes. We hypothesized that a decreased number of macrophages in the MG thymus could alter the efferocytosis efficacy of apoptotic thymocytes upon acute thymic stress. This would induce the abnormal IFN-I signature and lead to thymic changes associated with MG.

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Author contributions

CP, O-MF, and AY performed experiments. FT collected samples and managed patient information in the database. VB and DD performed Simoa experiments. SD provided blood samples. EF and DG provided thymic samples. FM and SB-A revised the manuscript. CP and RLP designed the study, analyzed the experiments, and wrote the manuscript.

Ethics approval and consent to participate

Studies on blood and thymic samples were approved by local ethics committees (RCB 2006-A00164-47 and RCB 2010-A00250-39) and informed consent forms were collected.

Competing interests

The authors declare that they have no competing interests.

Figure legends

Figure 1. IFN-I signature in serum and PBMC of MG patients. Measure of serum levels of IFN- β by ELISA (A) and IFN- α isoforms by Simoa (B) in MG patients (n=44) and healthy donors (n=14). mRNA expression of IFN- β (C), IFN- α 2 (D), IFNAR1 (E), and IFNAR2 (F) and several ISG, OAS1 (G), MX1 (H), ISG15 (I), IFIT1 (J), ISG12 (K), IFI44L (L), SIGLEC1 (M), and RSAD2 (N) was measured in PBMCs of MG patients (n=24) and healthy donors (n=11). mRNA expression was normalized on 28S. p values were determined by an ANOVA test with Dunn's post hoc tests and indicated only when $p < 0.05$ (*).

Figure 2. Effects of endogenous nucleic acids on TECs. mRNA expression of IFN- β (A) and α -AChR (B) by TECs measured 24h after stimulation with HSV-60 100 μ g/mL, CpG 5 μ g/mL, Poly(dA:dT) 100 μ g/mL, or 2'3'-cGAMP 100 μ g/mL. Representative time course effect of 2'3'-cGAMP on IFN- β (C) and α -AChR (D) mRNA expression by TECs. Effects of 2'3'-cGAMP (E) and Poly(dA:dT) (F) on α -AChR expression by TECs pretreated with or without 10 μ g/mL of an IFNAR2 blocking antibody by TEC. mRNA expression was normalized on GAPDH, and for each experiment, the mRNA level of untreated control cells was fixed at 100. p values were determined by ANOVA test with Dunn's post hoc tests for multiple comparisons or by the Mann-Whitney test for 2-by-2 comparisons. p values were indicated only when < 0.05 ($p < 0.05$ (*) and $p < 0.0001$ (***)).

Figure 3. Analysis of the effects of necrotic thymocytes on TECs. Measurement of thymocyte apoptosis/necrosis before (A) and after (B) a 4h dexamethasone treatment by FACS with Annexin V and PI labeling. mRNA expression of IFN- β (C), α -AChR (D), β -AChR (E), and ϵ -AChR (F) by TECs after co-culture with apoptotic/necrotic thymocytes. mRNA expression was normalized on GAPDH, and for each experiment, the mRNA level of untreated control cells was fixed at 100. p values were obtained by ANOVA test with Dunn's post hoc tests and indicated only when $p < 0.05$ (** $p < 0.001$).

Figure 4. In vivo analyses of the effects of necrotic thymocytes in the thymus of mice. Mice were injected with dexamethasone 5mg/kg/injection every other day. After a week, mice were sacrificed. The thymuses were weighted (A) and the absolute number of thymocytes was assessed (B) by FACS.

mRNA expression of keratin 5 was measured and normalized on GAPDH (C). The percentages of apoptotic (D) and necrotic (E) thymocytes were measured by FACS with Annexin V and PI labeling. Analysis of mRNA expression of IFN- β (F), α -AChR (G), IFN- α 2 (H), and β -AChR (I) in the thymus of injected mice. mRNA expression was normalized on GAPDH. Correlations of IFN- β (J) or IFN- α 2 mRNA (K) expression with α -AChR expression were analyzed. p values were obtained by the Mann-Whitney test for 2-by-2 comparisons and by the Spearman test for correlation (*p<0.05, **p<0.001, ***p<0.0001, ****p<0.00001).

Figure 5. Analyses of thymic macrophages in MG patients and the effect of a decreased number of thymic macrophages in mice. By immunofluorescence, the number of CD68⁺ cells was quantified in the thymus of MG patients and healthy donors (A-B). Mice were injected with an anti-CSF1R antibody to decrease thymic macrophages. After four weeks, mice were sacrificed. The thymuses were weighted (C) and the absolute number of thymocytes was assessed (D) by FACS. mRNA expression of keratin 5 was measured and normalized on GAPDH (D). The percentages of apoptotic (E) or necrotic (F) thymocytes were measured by FACS with Annexin V and PI labeling. mRNA expression of IFN- β (E), α -AChR (F), IFN- α 2 (G), and β -AChR (H) was measured and normalized on GAPDH. p values were obtained by the Mann-Whitney test for 2-by-2 comparisons (*p<0.05, **p<0.001).

Fig1. IFN-I signature in serum and PBMC of MG patients

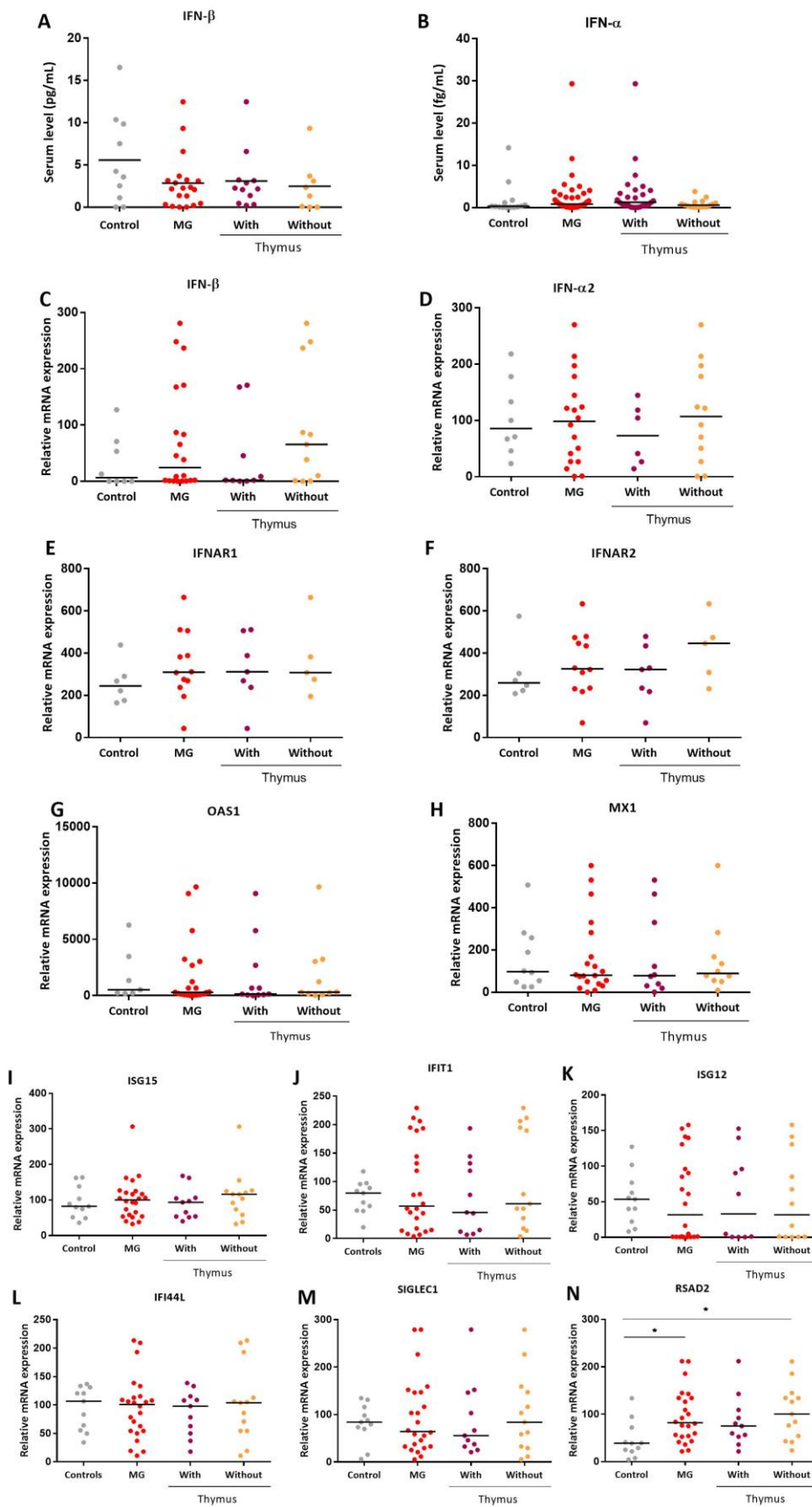


Fig2. Effects of endogenous nucleic acids on TECs

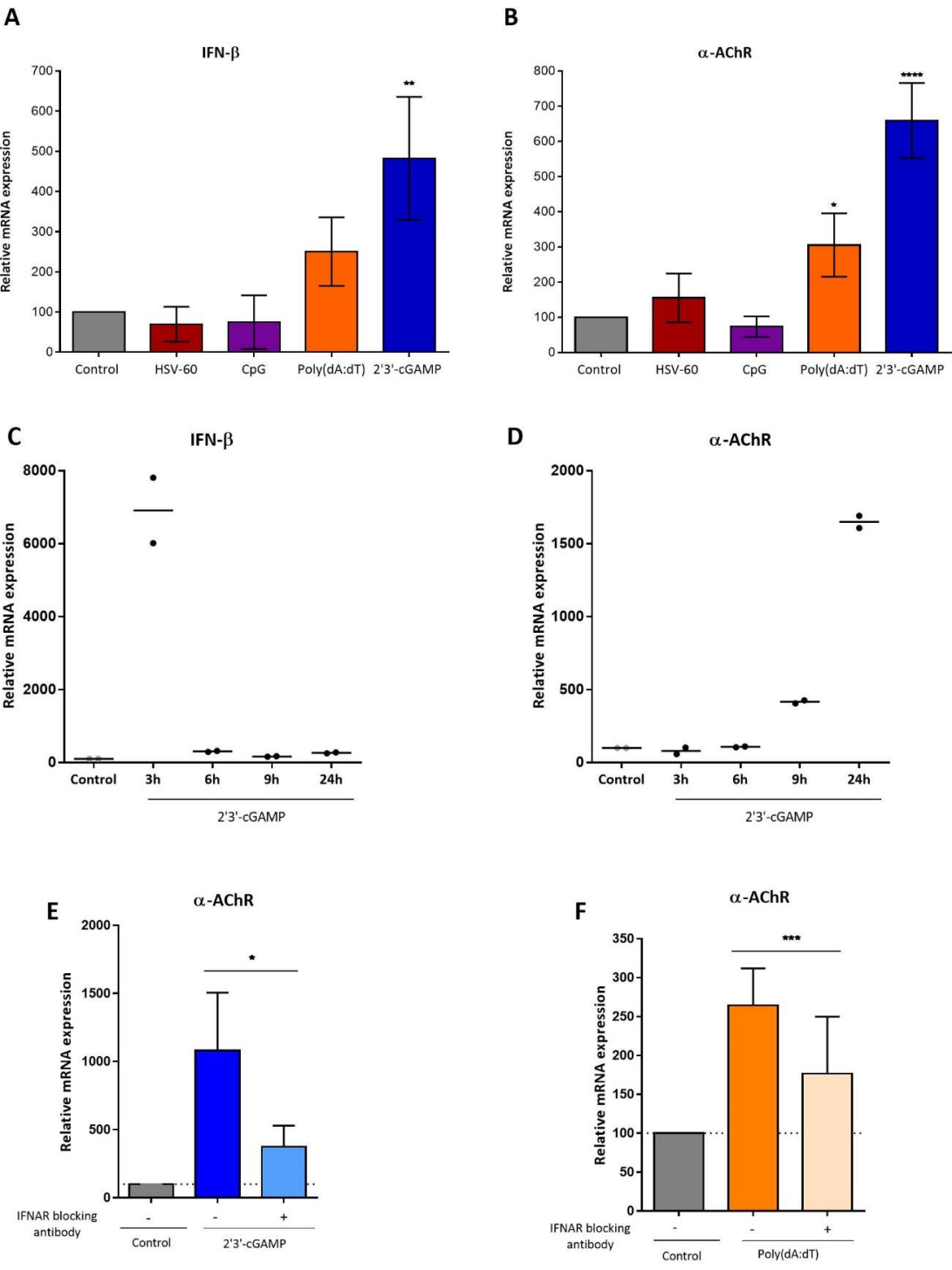


Fig3. Analysis of the effects of necrotic thymocytes on TECs

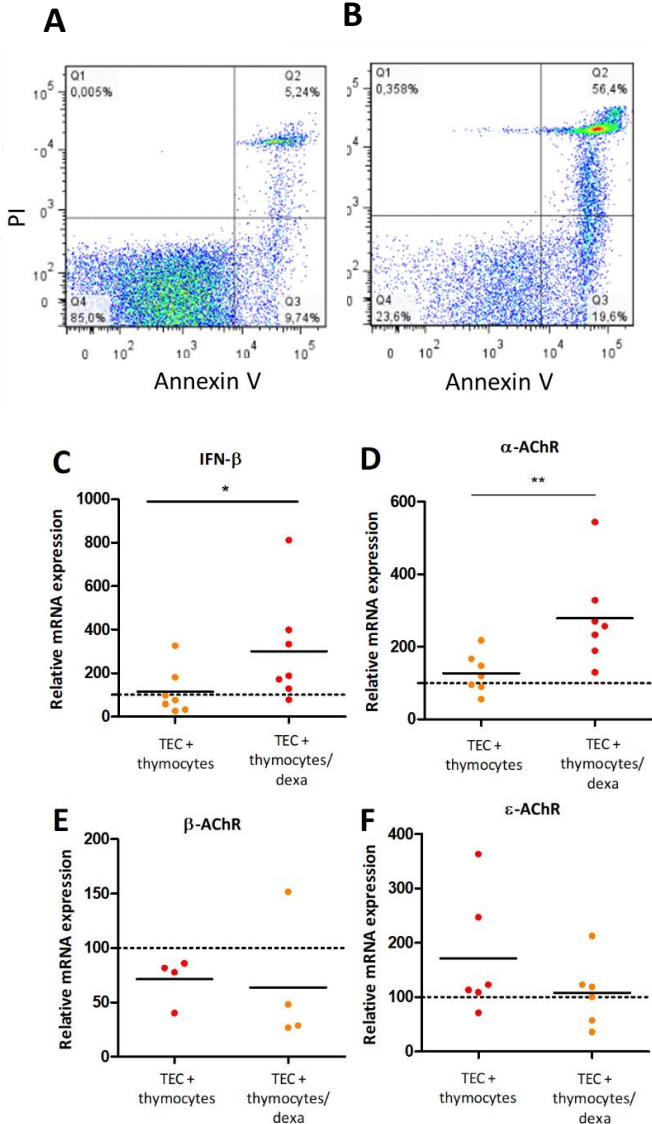


Fig4. In vivo analyses of the effects of necrotic thymocytes in the thymus of mice.

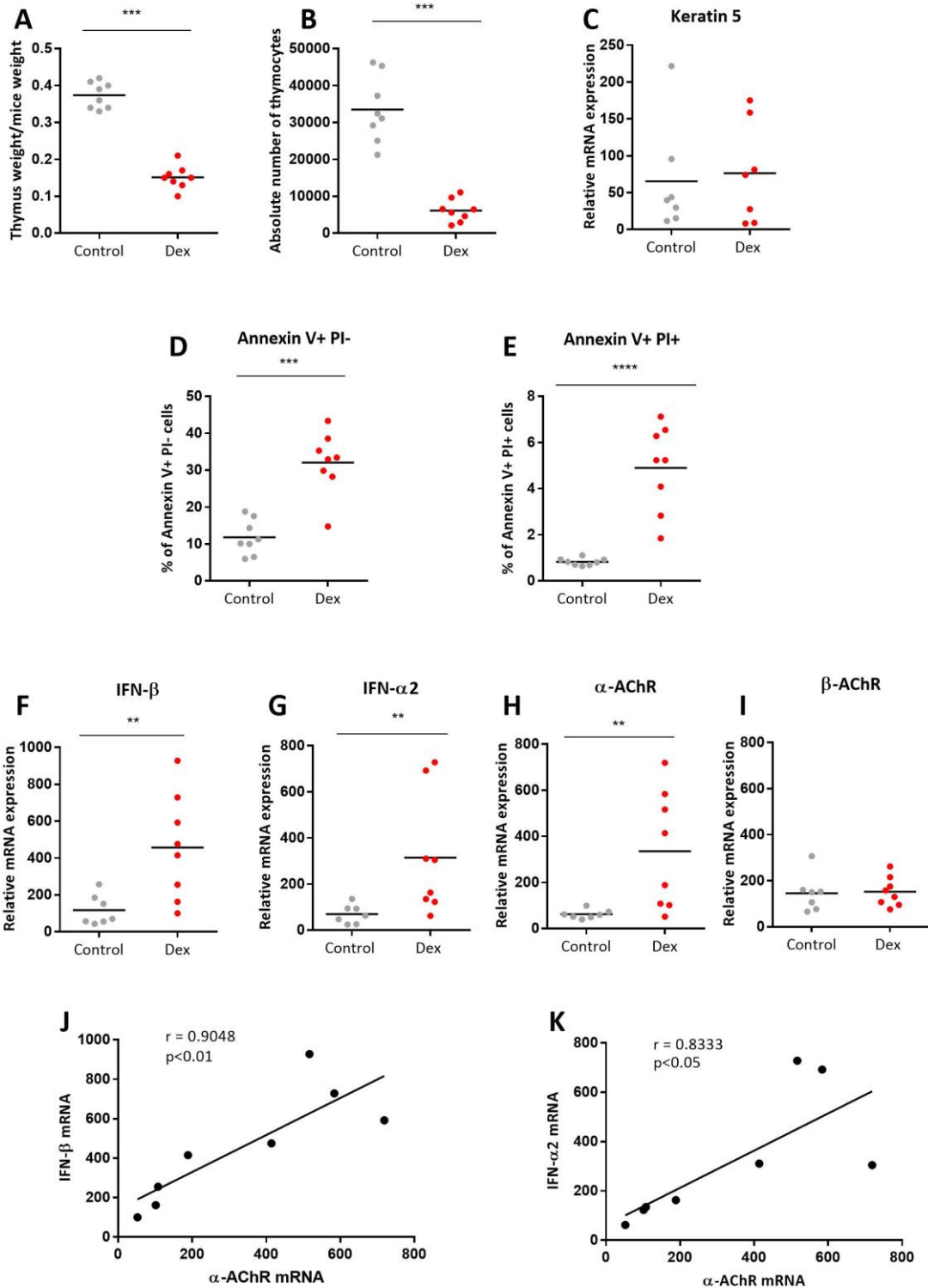


Fig5. Analyses of thymic macrophages in MG patients and the effect of a decreased number of thymic macrophages in mice.

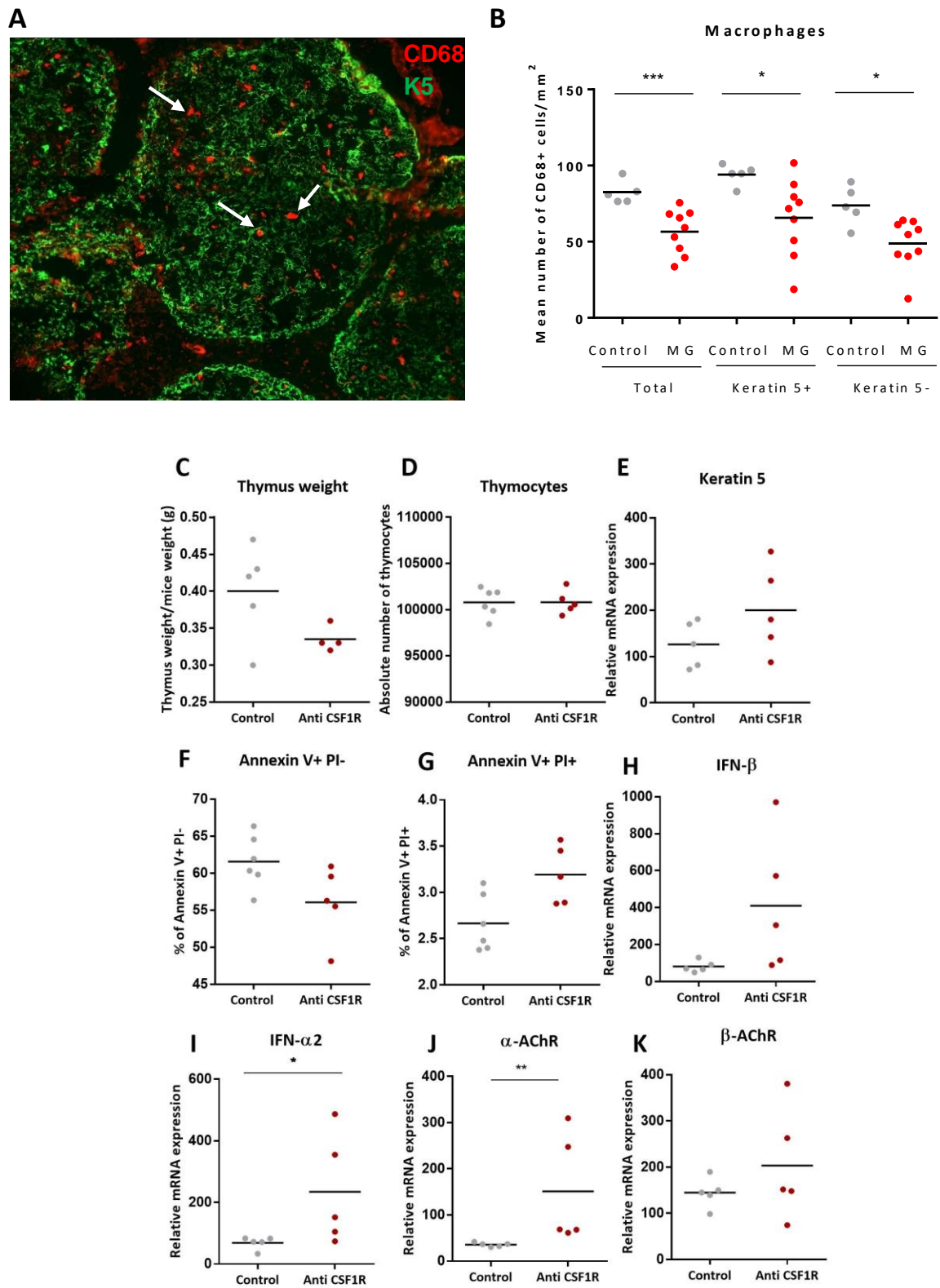
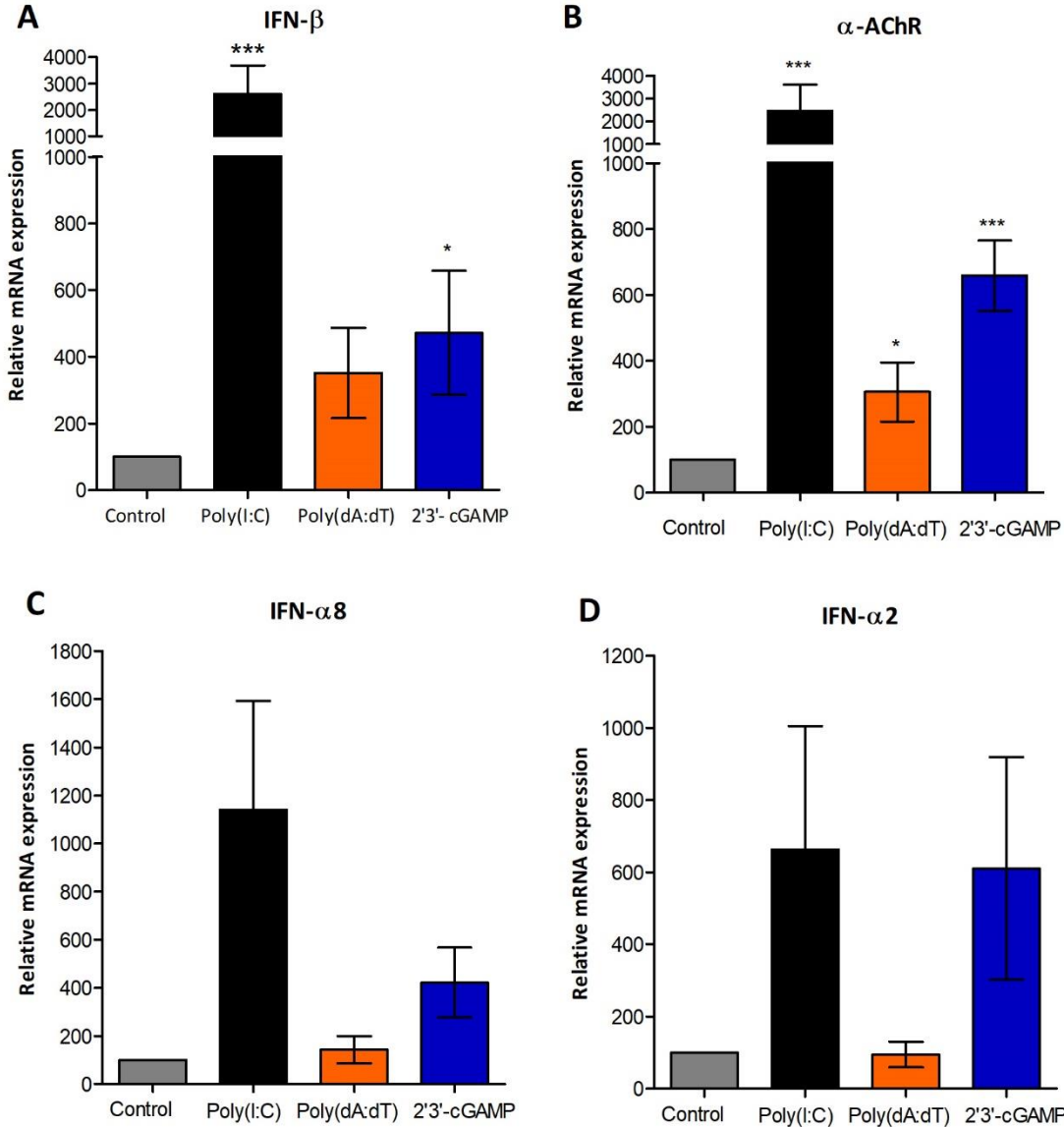
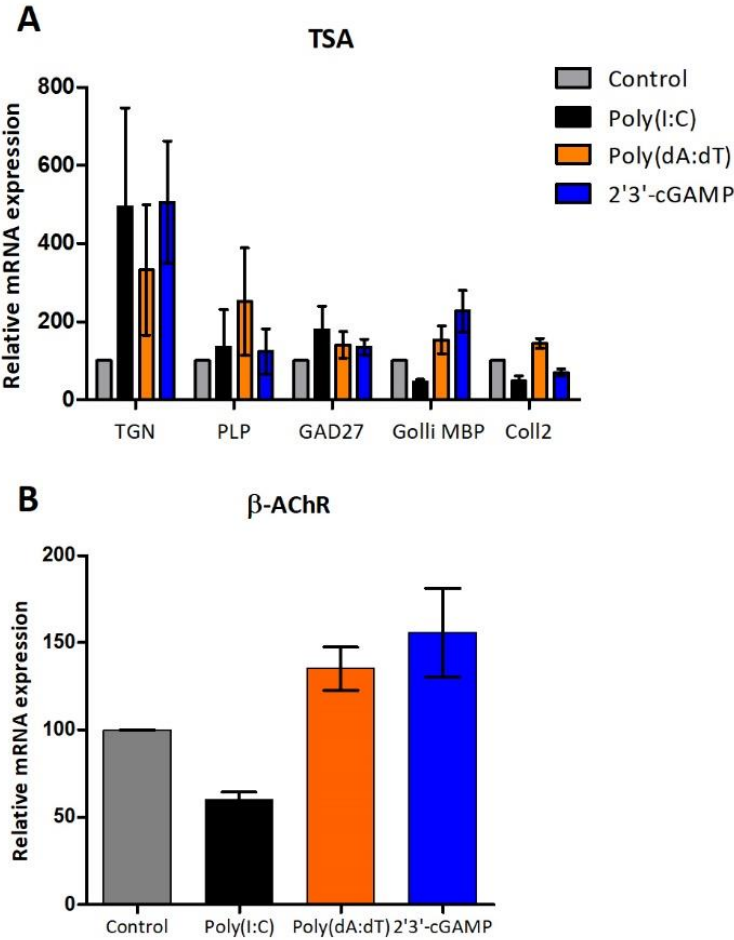


Figure S1. Analysis of Poly(I:C) effect on TECs



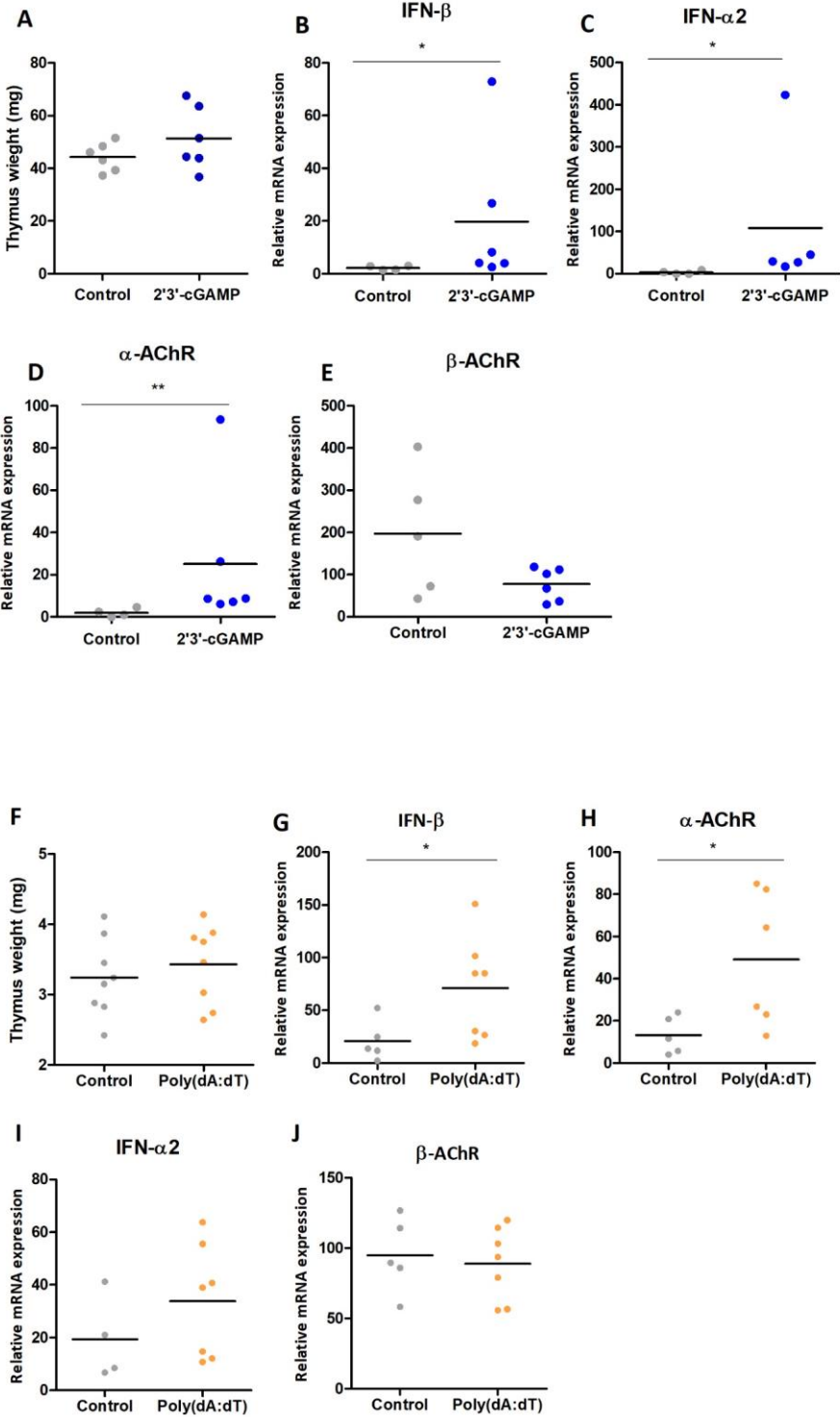
mRNA expression of IFN-β (A), α-AChR (B), IFN-α2 (C) and IFN-α8 (D) by TECs were measured after a 24h stimulation with Poly(I:C) 100µg/mL, Poly(dA:dT) 100µg/mL or 2'3'-cGAMP 100µg/mL. mRNA expression was normalized on GAPDH, and for each experiment the mRNA level of untreated control cells was fixed at 100. p values were obtained by ANOVA test with Dunn's post hoc tests for multiple comparisons (*p<0.05, ***p<0.0001).

Figure S2. Effects of endogenous nucleic acids on tissue specific antigen expression by TECs



mRNA expression of tissue specific antigens by TECs upon treatment with Poly(I:C) 100 μ g/mL, Poly(dA:dT) 100 μ g/mL or 2'3'-cGAMP 100 μ g/mL. mRNA expression was measured and normalized on GAPDH. For each experiment the mRNA level of untreated control cells was fixed at 100. p values were obtained by ANOVA test with Dunn's post hoc tests for multiple comparisons (* p <0.05, ** p <0.001).

Figure S3. *In vivo* analyses of the effects of 2'3'-cGAMP and Poly(dA:dT) on the thymus of mice



mRNA expression of tissue specific antigens by TECs upon treatment with Poly(I:C) 100µg/mL, Poly(dA:dT) 100µg/mL or 2'3'-cGAMP 100µg/mL. mRNA expression was measured and normalized on GAPDH. For each experiment the mRNA level of untreated control cells was fixed at 100. p values were obtained by ANOVA test with Dunn's post hoc tests for multiple comparisons (* $p < 0.05$, ** $p < 0.001$).

Table 1. Primers sequences

	Gene name	Sens primer	Antisens primer
Human primers	<i>28S</i>	CGGGTAAACGGCGGGAGTAA	GGTAGGGACAGTGGGAATCT
	<i>α-AChR</i>	AAGCTACTGTGAGATCATCGTCAC	TGACGAAGTGGTAGGTGATGTCCA
	<i>β-AChR</i>	ACAGCTACGACAGCTCGGAG	AGCGGCGGATCATGAGGTAG
	<i>Collagen II</i>	TCACGTACACTGCCCTGAAG	TGCAACGGATTGTGTTGTTT
	<i>GAD27</i>	CACAAGGTGGCTCCAAAAAT	TTACAGATCCTGGCCCAGTC
	<i>GAPDH</i>	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG
	<i>Golli MBP</i>	AAACCACGCAGGCAAACG	AGGGTCTCTTCTGTGACG
	<i>IFI44L</i>	GGTGTGCCGCAATGAGAGTC	ATGGAGGGGTGGTTTGCAGT
	<i>IFIT1</i>	TGGTGACCTGGGGCAACTTT	AGGCCTTGGCCCCTTCATAA
	<i>IFN-α2</i>	TCCTGCTTGAAGGACAGACA	TTTCAGCCTTTTGGAACTGG
	<i>IFN-α8</i>	CTCCTGCCTGAAGGACAGAC	AGCTGCTGGTCAAGTTCGAT
	<i>IFN-β</i>	ACGCCGCATTGACCATCTATG	CGGAGGTAACCTGTAAGTCTGT
	<i>IFNAR1</i>	CCTCCTGTGAGCCTAAGTGC	AAGGGCCTACCCTCAGTGTT
	<i>IFNAR2</i>	GAGCACAGTGATGAGCAAGC	TCAAGACTTTGGGGAGGCTA
	<i>ISG15</i>	CCACCTGAAGCAGCAAGTGA	ATTTCCGGCCCTTGATCCTG
	<i>ISG12</i>	GCCTCTGCTCTCCCTCATC	ATCTTGGCTGCTATGGAGGA
	<i>Mxa</i>	ACCTACAGCTGGCTCCTGAA	CGGCTAACGGATAAGCAGAG
	<i>OAS1</i>	CAAGCTCAAGAGCCTCATCC	TGGGCTGTGTTGAAATGTGT
	<i>PLP</i>	CTTCAACACCTGGACCACCT	GTGTTCTCCCATGGAATGCT
	<i>RSAD2</i>	TGGGAGGACAATGTGGGTGG	TGGCGCTATCTTGGCTCTGT
<i>TGN</i>	CCTGTGAGCAGACACCTGAA	GAGCCCTTTGCTTTTCACAG	
<i>SIGLEC1</i>	ATCGTTGGTATCGGGATGGC	GGGTAGTGAGTGTGACGTGG	
Mouse primers	<i>α-Achr</i>	GTGCTGGGCTCTTTCATCTC	TTCTGTGCGCGTTCTCATAAC
	<i>β-Achr</i>	GGACCGTCTTTTTCTGTGGA	GATTCTTACAAGCGCCAAGC
	<i>ε-Achr</i>	GAGGGAGGATATGTGAGCTG	ATCATGCCTGGGCAGTAGTC
	<i>Gapdh</i>	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
	<i>Ifn-α2</i>	TCTGTGCTTTCCTCGTGATG	TTGAGCCTTCTGGATCTGCT
	<i>Ifn-β</i>	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTTCA
	<i>Keratin 5</i>	TCAAGAAGCAGTGTGCCAAC	TCCAGCAGCTTCCTGTAGGT

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Discussion

An IFN-I signature is clearly detected in the thymus of MG patients in both the early-onset form of the disease (Cufi et al., 2013) but also in thymoma-associated MG (**ARTICLE 2 IN ANNEX: (Lefeuve et al., 2020)**). This clearly links IFN-I with the self-sensitization against AChR, as no thymic changes associated with IFN-I have been observed in MuSK MG patients. The thymic IFN-I signature observed in EOMG is associated mainly with the overexpression of IFN- β . In fact, IFN- β is the orchestrator of thymic changes. It induces the expression of CXCL13 and CCL21, the chemokine responsible for peripheral cell recruitment in the thymus but also BAFF, a pro-B cell survivor cytokine. The thymus becomes a favorable environment for B cell recruitment and formation of ectopic germinal center, leading to autoantibody production. Moreover, IFN-I increases the expression of the pro-inflammatory cytokine IL-23 by TECs, inducing T cell differentiation toward pathogenic Th17 cells, sustaining thymic inflammation (Villegas 2019). Furthermore, IFN- β decreases miR29 expression, which also favors the emergence of Th17 cells (Cron et al., 2018).

The objective of my PhD project was to study the IFN-I signature in EOMG in the periphery and the thymus to better understand its implication in the pathogenesis of the disease.

Could MG be related to interferonopathies?

Viral infection leads to an acute IFN-I signature to eliminate the virus. In MG, the IFN-I signature in the thymus is found even long after the disease onset, suggesting chronic IFN-I expression, as observed in interferonopathies. Interferonopathies are characterized by an overexpression of IFN-I in the periphery and auto-inflammation (Crow, 2011). Autoimmune non-genetic diseases are also part of this interferonopathy family, such as SLE and dermatomyositis (Baechler et al., 2011; Rodero and Crow, 2016). I searched for an IFN-I signature in the serum and PBMC of MG patients, and as the MG thymus overexpressed IFN- β , I investigated whether an IFN-I signature could be observed in the periphery in non-thymectomized patients as compared to thymectomized patients. This part of my PhD took me almost a year between the collection and sorting out of samples and the data analysis. No overexpression of IFN-I or ISG was detected when MG patients were analyzed as a whole but also

separately according to their thymectomy status. For another team project, I also demonstrated that thymoma-associated-MG (MGT) does not overexpress IFN-I subtypes in the periphery (**ARTICLE 2 IN ANNEX: (Lefevre et al., 2020)**). Nevertheless, it will be interesting to assess the IFN-I signature at disease onset to see if a transient peripheral IFN-I signature could be involved in the establishment of the disease. My results showed that MG is distinct from the other autoimmune diseases recently linked to interferonopathies. These autoimmune diseases with an IFN-I signature in the periphery but also in specific organs are systemic autoimmune diseases. In addition, they are characterized by the presence of anti-nuclear antibodies (Sur et al., 2018). No obvious anti-nuclear antibody signature is detected in MG. MG is a prototypical organ-specific autoimmune disease for which the target organ is the neuromuscular junction skeletal muscles. Of note, the muscle is not inflammatory and no IFN-I signature has never been observed in MG muscle (Maurer et al., 2015). In this context, we could consider that MG is thymic-specific interferonopathy. In MG, the thymus is the effector organ, which suggests that the IFN-I signature is associated with the pathogenesis of the disease.

How can we explain the thymic IFN-I signature in MG?

The obvious explanation for the IFN-I signature in the thymus of MG patients is viral infection. However, a recent review of several studies about the possibility of infectious agents in MG establishments failed to demonstrate a correlation between MG and infection (Leopardi et al., 2021). Moreover, no specific pathogen signature was discovered by the team when they analyzed thymic extracts from MG patients using virochips (Palacios et al., 2007) (collaborative project with Prof. Ian Lipkin, data not shown). Usually, IFN-I is produced after viral nucleic acids (NA) are sensed but endogenous nucleic acids (eNA) can be recognized by the same receptors and induce IFN-I production (Roers et al., 2016). Therefore, during my PhD project, I investigated the potential implication of eNA in thymic IFN- β production. I demonstrated that a molecule mimicking endogenous dsDNA (Poly(dA:dT)) and a molecule produced in response to dsDNA (2'3'-cGAMP) were able to induce IFN- β by TEC as well as α -AChR. Blocking IFN-I signalization inhibited Poly(dA:dT) and

2'3'-cGAMP effects on α -AChR expression. Other molecules mimicking dsDNA from bacteria or viruses failed to induce IFN- β and α -AChR.

To sustain these *in vitro* results, I analyzed *in vivo* the effects of Poly(dA:dT) or 2'3'-cGAMP in mice and studied thymic changes. These two molecules triggered the overexpression of IFN-I subtypes and α -AChR in the thymus of treated mice, as we found *in vitro*. In fact, the same *in vitro* and *in vivo* results were observed when Poly(I:C) was used (Cufi et al., 2013, 2014c). Poly(I:C) is a synthetic molecule mimicking viral or endogenous dsRNA and a strong inducer of IFN-I. In physiological conditions, eNA are not supposed to be recognized by sensors, and many degrading or compartmentalization mechanisms are in place to prevent inappropriate detection (Barton and Kagan, 2009). However, genetic polymorphism or mutations can induce a tolerance breach and eNA can be sensed by the same sensors as those detecting NA from pathogens (Miyake and Kaisho, 2014). In interferonopathies, eNA are involved in IFN-I production. Patients carried mutations on multiple genes, including genes implicated in the degradation of eNA (Crow, 2015). Accumulation of eNA is then mistaken as non-self NA and induces an IFN-I response. Previous results obtained by the team showing that Poly(I:C) induces IFN-I and α -AChR in TEC cultures and in the mouse thymus were thought to be due to the property of Poly(I:C) to mimic viral dsRNA (Cufi 2013). However, Poly(I:C) can also mimic endogenous dsRNA.

Altogether, these results demonstrate that molecules mimicking endogenous NA can induce an IFN-I signature in the thymus, leading to an upregulation of α -AChR in TEC.

Where could endogenous nucleic acids come from?

Different sources of eNA could be responsible for an IFN-I signature. Among the eNA are retroelement DNA, metabolites produced by dsRNA degradation, and mitochondrial DNA. Alternatively, eNA can derive from necrotic cells that can release their intracellular content (Uggenti et al., 2019).

A primary source of dsRNA structures in human cells is RNA produced by endogenous retro-elements. Long interspersed nuclear element (LINE-1 for 10% of endogenous dsRNA) or short

interspersed nuclear element (SINE for 80% of endogenous dsRNA) can be implicated in the induction of IFN-I response in humans. Retro-elements originate from retroviruses and integrated the human genome millions of years ago (Hancks and Kazazian, 2012). Nearly half of the genome is composed of noncoding retro-elements but only a small proportion is active. Nevertheless, these retro-elements, as a source of dsRNA, can activate innate signaling pathways. Mavragani et al. observed an increased expression of LINE-1 mRNA that correlates with the expression of IFN-I in SLE and primary Sjögren's syndrome (Mavragani et al., 2016). We analyzed the level of LINE-1 RNA in the thymus of MG patients but did not observe any differences (data not shown). Heinrich et al. also showed that Alu RNA elements stimulate the IFN-I response in multiple sclerosis (Heinrich et al., 2019). Alu RNA element can activate the MDA5-MAVS-IFN-I signaling pathway if not edited by the enzyme dsRNA degrading enzyme ADAR1 (Ahmad et al., 2018). Recently, Tossberg et al. observed that ADAR1 is decreased in multiple sclerosis and that unedited Alu RNA are potent inducers of the IFN-I signature and the NF- κ B signaling pathways (Tossberg et al., 2020). Interestingly, we observed a decreased expression of ADAR in the thymus of MG patients (data not shown), which should be further investigated.

In physiological conditions, genetic material is confined to cells, and systems are in place to avoid the leaking of endogenous DNA or RNA. However, non-processed apoptotic cells can progress to a secondary necrosis stage, resulting in the release of cell content due to the loss of plasma and nuclear cell membrane integrity (Zhang et al., 2018). Therefore, we wonder if necrotic cells could be the source of endogenous NA involved in the thymic IFN-I signature. I showed that necrotic thymocytes induce the production of IFN- β and that of α -AChR when co-cultured with TEC. I investigated *in vivo* the potential effect of acute thymic stress associated with a huge increase in thymocyte apoptosis/necrosis on the thymic IFN-I signature. Mice were injected with dexamethasone, a well-known thymocyte apoptosis inducer (E van Vliet and M Melis, 1986). As expected, an increase in apoptosis/necrosis thymocytes was detected and clearly associated with an IFN-I signature and the increased expression of α -AChR.

In the case of an acute inflammatory event, RNA from necrotic cells can activate TLR3 at the cell surface and exacerbate the immune response (Cavassani et al., 2008). Interestingly, TLR3 is overexpressed in the thymus of patients and could drive over-inflammation in response to RNA from necrotic cells.

Moreover, the accumulation of DNA from necrotic cells is normally degraded by DNase I and DNase II in tissues and serum to avoid autoinflammation (Napirei et al., 2000). Indeed, some SLE patients carried a mutation of DNase, which can be linked to the accumulation of DNA released from apoptotic cells, triggering an autoimmune response (Al-Mayouf et al., 2011; Yasutomo et al., 2001).

I demonstrated that acute thymic stress, by inducing thymocyte apoptosis/necrosis, can induce an IFN-I signature in the thymus, leading to an upregulation of α -AChR in TEC and recapitulate some features found in the thymus of MG patients.

What is the implication of macrophages in nucleic acid accumulation?

After demonstrating that apoptotic/necrotic cells could be implicated in the IFN-I signature in MG, we wonder why thymocytes could become necrotic and release their content. In physiology, apoptotic cells are phagocytosed by different cell populations but mainly macrophages. This process is called efferocytosis and is considered “immunologically silent” (Green et al., 2016). It prevents apoptotic cells from progressing to a secondary necrotic stage. Actually, efferocytosis is important for the resolution of inflammation by restraining the production of pro-inflammatory cytokines and increasing the production of anti-inflammatory molecules (Kasikara et al., 2018). Therefore, we studied thymic macrophages in MG. By quantification, I showed for the first time that MG patients display fewer thymic macrophages compared to healthy controls. We do not know if the decreased number of macrophages is a cause or consequence of MG but in this research project, we hypothesized that an alteration in macrophages could be part of the cause of the disease. Indeed, a defect in thymic macrophages could alter the efficient efferocytosis of apoptotic thymocytes, leading to an increased proportion of necrotic thymocytes and the release of eNA. To test this hypothesis, I used an animal model deficient for macrophages. It was difficult to find the most

appropriate model because few studies looked at thymic macrophages after inducing macrophage deficiency. We decided to use an inducible model by injected antibodies against CSF1R (Sauter et al., 2014). CSF1 is a cytokine responsible for the proliferation, differentiation, maturation, and survival of macrophages (Chitu and Stanley, 2006). Autoantibodies block the binding between the cytokine and its receptor and prevent the replenishment of tissue macrophages. In our experiments, injected mice had more necrotic cells and, conversely, a tendency toward less apoptotic cells, proving that the decrease in macrophages could induce an accumulation of necrotic cells. Moreover, the thymuses of injected mice displayed an IFN-I signature and an increased expression of α -AChR. Leakage of cell content and, therefore, eNA could be responsible for overexpression of IFN-I in the thymus of injected mice and perhaps in the MG thymus.

In fact, several autoimmune diseases showed implication deficiency in efferocytosis, such as SLE, MS, Sjögren's syndrome, Type 1 diabetes, and others. These diseases showed that the inflammation is, in part, due to an altered clearance of apoptotic cells, inducing the release of harmful content (Abdolmaleki et al., 2018). Efferocytosis is a process with different steps: 1) find-me and eat-me molecules are sent by apoptotic cells to attract phagocytes, 2) the apoptotic cell is engulfed by phagocytes, and 3) the phagocytosed cell is processed. Depending on the disease, the patient displays abnormalities at different steps of the efferocytosis process (Green et al., 2016). We wonder if, in addition to a decreased number, thymic macrophages from MG patients could be less efficient for efferocytosis. This would need to be further investigated.

What could explain the chronic inflammation observed in the MG thymus?

Altogether, the results of my PhD suggest that a decreased number of thymic macrophages could initiate the chronic inflammation observed in the MG thymus. This could occur upon an acute thymic involution due to stress, viral infection, cancer, or pregnancy (Dooley and Liston, 2012). Nevertheless, upon acute involution, the thymus is known to rapidly recover (Ansari and Liu, 2017) and the resulting inflammation is rapidly calmed down. We thus wonder if a decrease in the ability of the thymic macrophage to eliminate apoptotic thymocytes is sufficient to explain the chronic IFN-I

signature observed in the MG thymus. We suspect that a factor affecting the resolution of thymic inflammation could be associated. Though MG is not a genetic disease, some gene polymorphisms could be implicated, notably, on genes regulating dsRNA or dsDNA processing. ADAR is found to be decreased in the thymus of MG patients, and loss of function of ADAR1 is also linked to the severe autoimmune disease AGS, an interferonopathy. Moreover, Nakahama et al. showed that ADAR1 is overexpressed in the thymus of adult mice and is important for T cell maturation, especially negative selection, which is important to prevent autoimmunity (Nakahama et al., 2018). The study also showed a diminution of regulatory T cells in mice lacking *adar*. The diminution in the MG thymus could participate in an abnormal T cell education, resulting in autoantibodies against α -AChR.

Moreover, a diminution of DICER, an enzyme implicated in miRNA processing but also in the processing of endogenous dsRNA, is found in the thymus of MG patients (**ARTICLE 3 IN ANNEX: (Cron et al., 2020)**)(Cron et al., 2020; White et al., 2014). This decrease could be part of the pathogenesis of MG by an altered processing of miRNA, leading to the accumulation of dsRNA. This could explain the IFN-I overexpression in the thymus of MG patients. Concomitant with the decrease in DICER, miR-29a is found downregulated in the thymus of MG patients (**ARTICLE 3 IN ANNEX: (Cron et al., 2020)**). Papadopoulou et al. showed that DICER is important to protecting thymic architecture, avoiding premature involution but also that miR-29a is implicated in the modulation of *ifnar1* (Papadopoulou et al., 2012). Therefore, a decrease in miR-29a could lead to an overexpression of IFNAR1 and increased IFN-I signalization, inhibiting the resolution of inflammation.

Another enzyme implicated in the degradation of NA is DNASEII. This enzyme is necessary for the degradation of phagocytosed cells in the context of efferocytosis. In fact, DNASEII is diminished in the thymus of MG patients but not significantly (data not shown).

Altogether, these results could explain a non-resolution of inflammation in the thymus of MG patients caused by endogenous AN.

Conclusion

All the results gathered during my PhD project help us to better define the thymic IFN-I signature in MG patients. First, I demonstrated that this signature was confined to the thymus and that MG is a kind of specific thymic interferonopathy. Next, by investigating the origin of this thymic IFN-I signature, I hypothesized that a triggering event (viral infection, stress, or pregnancy) leads to the massive apoptosis of thymocytes. Patients who display fewer thymic macrophages become overwhelmed with apoptotic cells. Non-processed apoptotic thymocytes turn necrotic and release their intracellular content, such as eNA. These molecules activate innate signaling pathways, leading to the IFN-I signature and the establishment of thymic changes. Moreover, we suspect that an abnormal resolution of the inflammation maintains the chronic IFN-I signature, even long after the triggering event. These PhD results have raised new questions about the implication of macrophages in the establishment of MG (a new ongoing PhD project in the team) and in the mechanisms involved in the resolution of thymic inflammation.

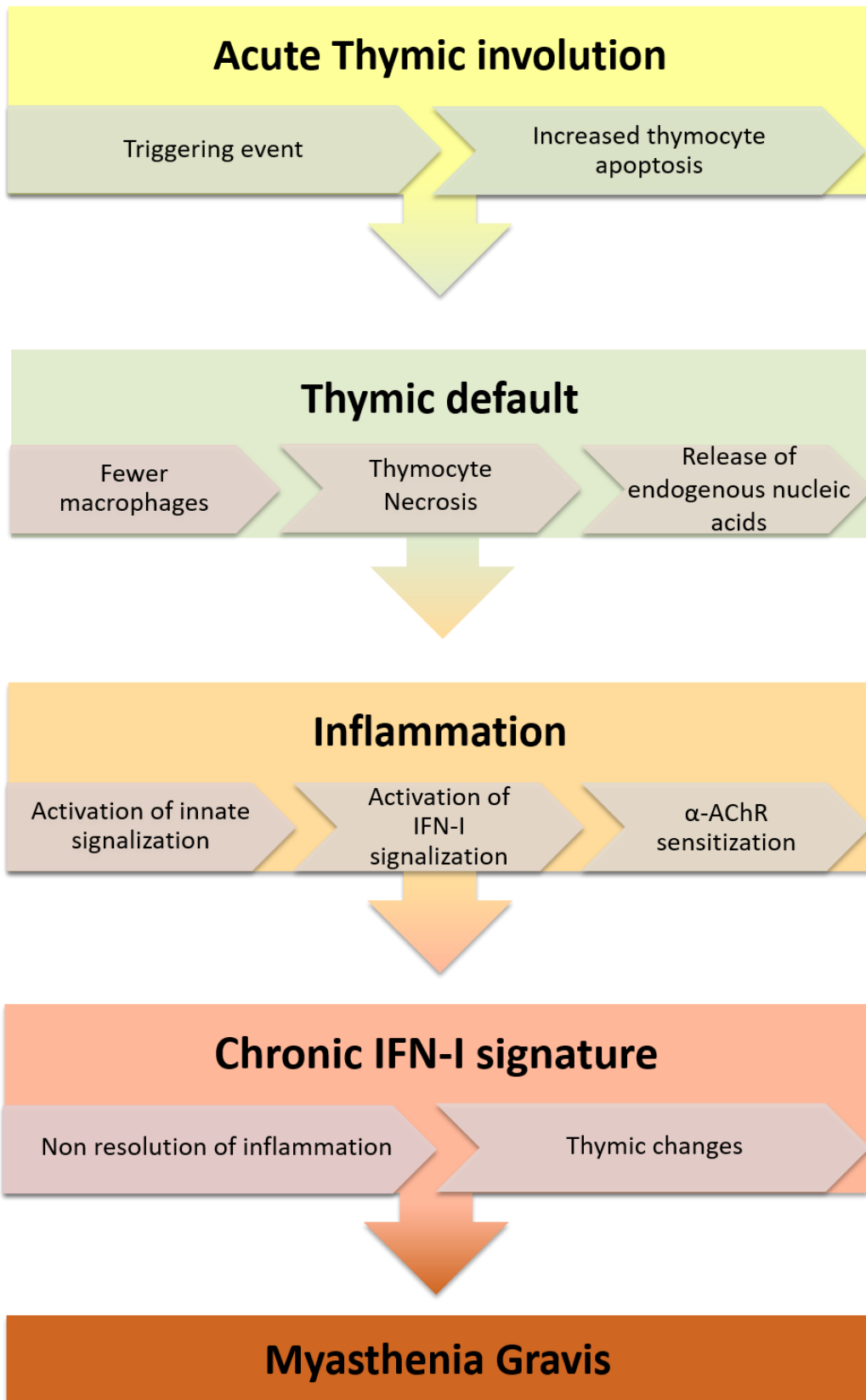


Figure 9: Summary figures of my PhD results

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Annexes

Article 2

Risk factors associated with myasthenia gravis in thymoma patients: The potential role of thymic germinal centers



Risk factors associated with myasthenia gravis in thymoma patients: The potential role of thymic germinal centers



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ABSTRACT

Thymomas are associated with a very high risk of developing Myasthenia Gravis (MG). Our objectives were to identify histological and biological parameters to allow early diagnosis of thymoma patients susceptible to developing MG. We conducted a detailed retrospective analysis from a patient database, searching for differences between patients with thymoma-associated MG (MGT, n = 409) and thymoma without MG (TOMA, n = 111) in comparison with nonthymomatous MG patients (MG, n = 1246). We also performed multiplex and single molecule arrays to measure the serum levels of cytokines in these groups of patients and controls (n = 14–22). We identified a set of parameters associated with MG development in thymoma patients: 1) detection of anti-acetylcholine receptor (AChR) antibodies, 2) development of B1 or B2 thymoma subtypes, 3) presence of ectopic thymic germinal centers (GCs), 4) local invasiveness of thymoma, and 5) being a woman under 50 years old. Among these parameters, 58.8% of MGT patients displayed GCs with a positive correlation between the number of GCs and anti-AChR titers. By immunohistochemistry, we found thymic GCs in the adjacent tissues of thymomas encircled by high endothelial venules (HEVs) that could favor peripheral cell recruitment. We also clearly associated MG symptoms with higher IFN- γ , IL-1 β and sCD40L serum levels, specifically in MGT patients compared to TOMA patients. Altogether, these analyses allowed the clear identification of histological, in particular the presence of GCs, and biological parameters that would facilitate the evaluation of the probability of the MG outcome postoperatively in thymoma patients.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction characterized by weakness and fatigability of skeletal muscles. MG is mediated by autoantibodies against proteins of the neuromuscular junction, mainly the acetylcholine receptor (AChR). Several classifications of MG patients are made based on 1) the autoantibody target, 2) the symptoms (ocular versus generalized

presentation) and their severity, 3) the age at onset (early onset form (EOMG) before 45–50 years old or late onset development after 50 years old), and 4) the association with a thymic pathology [1]. Indeed, thymic abnormalities are observed in AChR-MG patients [2]. Different thymic abnormalities are described in AChR-MG patients: 1) 85% of the patients, mainly females, have thymic follicular hyperplasia, which is characterized by B-cell infiltration leading to ectopic germinal center (GC) development [1]; and 2) 10–15% of MG patients have thymoma,

Abbreviations: AChR, acetylcholine receptor; EOMG, early-onset myasthenia gravis; GC, germinal center; HEV, high endothelial venule; IL, Interleukin; IFN, Interferon; MG, myasthenia gravis; MGT, thymoma-associated MG; MuSK, muscle-specific tyrosine kinase; sCD40L, soluble CD40 ligand; Tfh, T follicular helper; TOMA, thymoma without MG; WHO, world health organization

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predominantly developing after 50 years old without sex predominance [3].

Thymomas correspond to thymic epithelial cell neoplasms and are among the most frequent anterior mediastinal tumors in adults. Several paraneoplastic syndromes are associated with thymoma. The most common is MG, but others are also described, such as systemic lupus erythematosus, rheumatoid arthritis, neuromyotonia, vitiligo, or pemphigus [4,5]. For patients with a thymoma, MG incidence occurs in approximately 30% of cases, but this rate is highly variable from one study to another [6]. Symptoms in thymoma-associated MG (MGT) patients are usually more severe with a higher frequency of generalized disease with bulbar and respiratory symptoms, and patients need more immunosuppressive treatments relative to MG patients without thymoma [7]. MG symptoms allow earlier detection of a thymoma and favor thymoma patient's survival [8]. However, MG can develop after thymectomy. Here, we conducted a detailed retrospective analysis from our patients' database, searching for differences between MGT and thymoma without MG (TOMA) patients, in comparison with MG patients without thymoma. We also measured the serum levels of diverse cytokines in these different groups of patients to potentially identify predictive biomarkers for MG. This study aimed to identify risk factors for thymoma patients developing MG symptoms after thymectomy.

2. Materials & methods

2.1. Patients' database

This study is a retrospective analysis from a computerized and anonymized French database established by Dr. Sonia Berrih-Aknin in collaboration with the Marie Lannelongue Hospital (Le Plessis-Robinson, France), Cochin Hospital (Paris, France), Institut Mutualiste Montsouris (Paris, France), Raymond Poincaré Hospital (Garches, France), Pitié-Salpêtrière Hospital (Paris, France) and Hautepierre Hospital (Strasbourg, France). From 1990 to 2018, 1766 patients were registered in this database, and 3 groups of patients have been identified (Fig. 1): 1) thymoma patients without MG at the time of surgery

(TOMA, n = 111), 2) thymoma-associated MG patients (MGT; n = 409) and 3) MG patients without thymoma, (MG; n = 1246) (Fig. 1). Patients with a diagnosis of thymic carcinoma were excluded. Clinical and biological data included biographic data, thymic histology, anti-AChR antibody titer (serum titer measured from 90 days before and up to 8 days after thymectomy and considered positive (AChR⁺) when above 0.5 nmol/l), and immunosuppressive treatments. The diagnosis of MG was based on clinical, pharmacological and electrophysiological criteria (Table 1).

2.2. Immunohistochemistry on thymic sections

Thymic biopsies from MGT patients, either the thymoma (n = 7) or the adjacent thymic tissue (n = 7), were analyzed and compared to those from MG patients. Frozen thymic sections (7 µm) were fixed in ice-cold acetone for 20 min. Immunofluorescence staining was performed to label B cells and follicular dendritic cells with a FITC mouse antihuman CD21 (clone BL13, Beckman Coulter) and the epithelial network with an eFluor 615 mouse antihuman cytochrome c (clone AE1/AE3, eBioscience). High endothelial venules (HEVs) were labelled successively with a rat antimouse PNAd (peripheral node addressin) carbohydrate epitope (MECA 79, BD Pharmingen), a biotin mouse anti-rat IgM (clone G53-238, BD Pharmingen) and Alexa Fluor 350 streptavidin (Invitrogen). Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope.

2.3. Human serum samples

Blood was collected from TOMA (n = 15–20; mean age 59.2 ± 2.4 [age range 37–78]), MGT (n = 16–19; mean age 53.6 ± 2.2 [age range 41–74]), early-onset MG patients (EOMG, n = 14–15; mean age 28.2 ± 2.3 [age range 17–45]) and non-MG controls (n = 20–22; mean age 43.6 ± 2.4 [age range 17–69]). Blood was collected before thymectomy from MG, MGT and TOMA patients that were not treated with immunosuppressors. Blood from control healthy donors was from the French Blood Establishment (EFS). Blood was collected under a

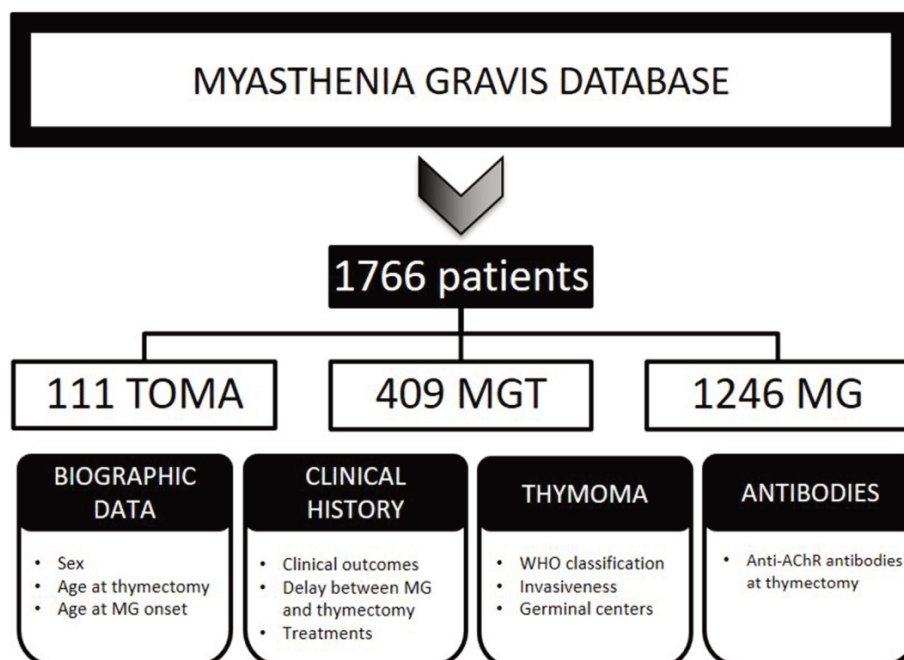


Fig. 1. Description of the parameters analyzed from the database.

Table 1
Characteristics of the patients included in the database.

	TOMA	MGT	MG
Patients (number of patients)	111	409	1246
Gender: number of female patients (%) ^a	55 (49.5%)	237 (57.9%)	913 (73.3%)
Age at MG onset in years (mean ± SEM) ^b	–	48.8 ± 0.7 (n = 356)	31.6 ± 0.4 (n = 1115)
Age at thymectomy in years (mean ± SEM) ^c	54.4 ± 1.3 (n = 105)	50.2 ± 0.7 (n = 405)	32.3 ± 0.4 (n = 1125)
Age range	17–89	9–89	12–62
Delay between MG onset and thymectomy in months (mean ± SEM) ^d	–	18.4 ± 1.9 (n = 353)	33.4 ± 1.6 (n = 1017)
Thymoma classification: number of patients (% for known patients) ^e			
A	17 (25.0%)	17 (5.1%)	–
AB	11 (16.2%)	31 (9.3%)	–
B1	15 (22.1%)	85 (25.5%)	–
B2	24 (34.8%)	182 (54.8%)	–
B3	2 (2.9%)	17 (5.1%)	–
Unknown	42	77	–
Invasive thymoma: number of patients (% for known patients) ^f			
Yes	40 (53.3%)	230 (70.6%)	–
No	35 (46.7%)	96 (29.4%)	–
Unknown	36	83	–
Immunosuppressive treatments: number of patients (% for all patients) ^g			
Yes	4 (3.6%)	83 (20.3%)	235 (18.9%)
None	107	326	1011
AChR antibodies: number of patients (% for known patients) ^h			
Positive at thymectomy	19 (51.3%)	261 (98.5%)	541 (76.6%)
Serum titer were measured from 90 days before and up to 8 days after thymectomy			
Negative at thymectomy	18 (48.7%)	4 (1.5%)	165 (23.4%)
Unknown	74	144	540
Presence of germinal centers: number of patients (% for known patients) ⁱ			
Yes	7 (15.6%)	161 (58.8%)	640 (64.5%)
None	38 (84.4%)	113 (41.2%)	352 (35.5%)
Unknown	66	135	254

^a Gender: TOMA vs MG $p < 0.0001$; MGT vs MG $p < 0.0001$ (Fisher's exact test).

^b Age at MG onset: MGT vs MG $p < 0.0001$ (Student t -test).

^c Age at thymectomy: TOMA vs MGT ($p < *$), TOMA vs MG ($p < ***$), MGT vs MG ($p < ***$) (one way anova test Bonferroni post hoc tests) - Fig. 2A.

^d Delay between MG onset and thymectomy: MGT vs MG $p < 0.0001$ (Student t -test) - Fig. 2B.

^e Thymoma WHO classification for TOMA vs MGT (comparison of each subtype versus all others for known patients): type A $p = 0.0001$ and type B2 $p = 0.002$ (Fisher's exact test).

^f Invasive thymoma for known patients independently from histological type: TOMA vs MGT $p = 0.0060$ (Fisher's exact test).

^g Immunosuppressive treatments (corticosteroids, azathioprine, mycophenolate mofetil, methotrexate, cyclophosphamide, rituximab): TOMA vs MGT or MG, p^{***} (Fisher's exact test).

^h Presence of anti-AChR antibodies: TOMA vs MGT or MG $p < 0.0001$, MGT vs MG $p < 0.0001$ (Fisher's exact test) - Fig. 3 for further investigations.

ⁱ Germinal centers for known patients: TOMA vs MGT or MG $p < 0.0001$ (Fisher's exact test).

vacuum container in a dried tube with separating gel (BD Vacutainer SST™ Advance Ref 367953) for coagulation and centrifugation. Serum samples were stored at -80°C until use. Studies on blood samples were approved by local ethics committees [RCB 2010-A00250-39].

2.4. Multiplex assays

Multiplex cytokine analyses were performed with the Bio-Plex Pro™ Human Th17 Cytokine Panel 15-plex (IL-1 β , IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IFN- γ , sCD40L, TNF- α) (BioRad, Marnes-La-Coquette, France) on an immunomonitoring platform (IMRB, VRI, INSERM U955 - UPEC, France). The samples were diluted 1:4 in the kit diluent and incubated overnight at room temperature before analysis. Differences in serum levels were analyzed using the fluorescence signals (minus the fluorescence background) as the serum levels were low and could not be determined precisely from the cytokine standard curves.

2.5. IFN- α measurement with Simoa assay

Serum levels of all interferon (IFN)- α subtypes were quantified using a single molecule array (Simoa) from Quanterix [9]. Each serum sample was diluted 1:3, and the limit of detection was calculated as the mean value + 2SD of reactivity from all blank runs and found to be 0.04 fg/ml.

2.6. Statistical analyses

GraphPad 5.0 software was used to perform statistical analyses and generate graphic representations. The results are expressed as the means and standard error of the mean (SEM) for the different

experiments. We used Student's t -test or the Mann-Whitney test for 2-by-2 comparisons, or the one way ANOVA with Bonferroni's Multiple Comparison Tests. Fisher's exact test was used for 2-by-2 table analyses and was performed using the online tool GraphPad QuickCalcs. The tests used are specified in the figures or table legends, and p -values are indicated on graphs when < 0.05 .

3. Results

3.1. Main characteristics of the patient cohort

We reviewed 1766 patients registered in the database with a clinical diagnosis of nonthymomatous MG (MG, $n = 1246$), or thymoma ($n = 520$) either associated with MG (MGT, $n = 409$) or without MG (TOMA, $n = 111$) (Fig. 1). From this database, 78.7% of thymoma patients had MG. The female/male sex ratios were 2.7 for MG, 1.4 for MGT and 1.0 for TOMA patients (Table 1). The proportion of female patients was significantly higher among MG patients relative to MGT and TOMA patients, and the proportion of female patients was also slightly higher among MGT than TOMA patients. As expected, non-thymomatous MG patients who develop symptoms at an early age were thymectomized at a younger age than patients with a thymoma (TOMA or MGT patients) (Fig. 2A). However, we also showed that MGT patients were thymectomized at a younger age than TOMA patients (Fig. 2A). In addition, MGT patients were thymectomized more rapidly after symptom onset than MG patients (Fig. 2B), probably because thymectomy must be processed rapidly once the thymoma is diagnosed [7].

The histological classification used is the one established by the World Health Organization (WHO), with type A, B1, B2, B3 and AB thymomas. Type A is often considered a medullary thymoma, types B1

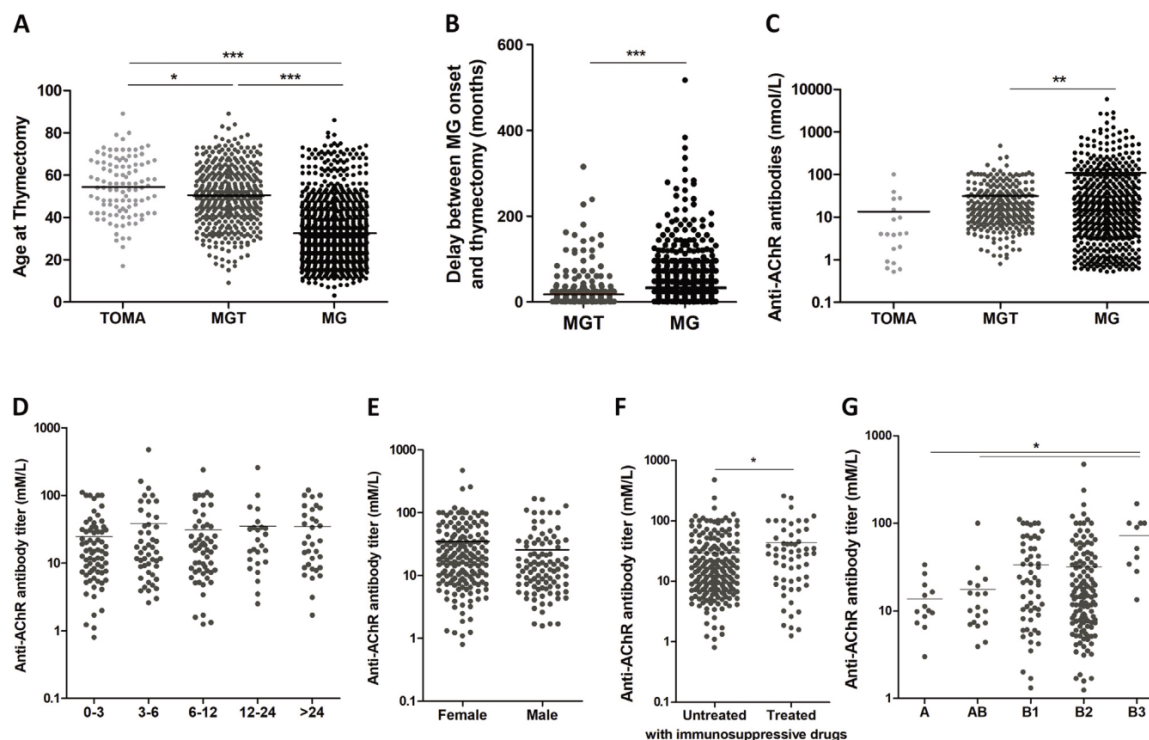


Fig. 2. Analyses of biological and clinical parameters in TOMA, MGT and MG patients. (A) Age of patients at the time of thymectomy for 105 TOMA, 405 MGT, and 1125 MG patients. (B) Delay between the onset of MG symptoms and thymectomy for 353 MGT, and 1017 MG. (C) Anti-AChR titer (> 0.5 nmol/l) for 19 TOMA, 261 MGT, and 541 MG patients. (D) Anti-AChR titers in AChR⁺ MGT patients according to delay between MG onset and thymectomy. (E) Anti-AChR titers in 165 females and 96 males in AChR⁺ MGT patients. (F) Anti-AChR titers in 208 untreated- and 58 treated-AChR⁺ MGT patients. (G) Anti-AChR titers in AChR⁺ MGT patients in different thymoma subtypes. p-values were assessed using the one-way ANOVA with Bonferroni post hoc tests (A, C, D, G) or the student t-test (B, E, F). p-values are indicated if $p < 0.05$ as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

and B2 are cortical thymomas, type AB is a mixed thymoma (involving both cortical and medullary epithelial cells) and type B3 is atypical thymomas. Here, more than 80% of the MGT patients displayed a B2 (54.8%) or B1 (25.5%) subtype. In the TOMA group, the proportions of the A, AB, B1 and B2 subtypes were more homogenous (25.0%, 16.2%, 22.1% and 34.8%, respectively). Comparing TOMA and MGT patients, significant differences were observed for the B2 subtype, which was highly represented in MGT patients, and the A subtype was more highly represented in TOMA patients (Table 1). Local invasiveness of the thymoma outside of the thymic capsule was significantly more frequent for the MGT group than the TOMA group (Table 1). As MGT patients were also treated with immunosuppressive drugs significantly more often (Table 1), we analyzed local invasiveness in treated and untreated patients. We observed that patients with immunosuppressive treatment more frequently have invasive tumors when patients were analyzed altogether (TOMA and MGT patients) or in MGT patients (data not shown). For TOMA patients, it was difficult to draw conclusions as only a very small number were treated with immunosuppressive drugs (data not shown).

We analyzed the anti-AChR antibody titer for all patients when measured before or at the time of thymectomy. A total of 76.6% of nonthymomatous MG patients had anti-AChR antibodies (Table 1). In 23.4% of seronegative patients, a few may have low-affinity anti-AChR antibodies that were not yet measurable with the appropriate cell-based assay in clinical laboratories in France, and the others could be positive for anti-muscle-specific tyrosine kinase (MuSK) or anti-LRP4 antibodies. In the MGT group, almost all patients (98.5%) were positive for anti-AChR antibodies (Table 1). This was not surprising as MGT is not associated with anti-MuSK or anti-LRP4 antibodies. In the TOMA group,

37 patients were analyzed for anti-AChR antibodies, and 51.3% were positive at thymectomy, suggesting that these patients were at high risk to develop MG symptoms after surgery. For all AChR⁺ patients, comparing the anti-AChR levels in the three groups of patients, we showed that MG patients had higher levels than MGT and TOMA patients, and MGT patients might also have higher levels than TOMA patients (Fig. 2C). Additionally, we analyzed the anti-AChR antibody titers in MGT patients according to different parameters. We did not observe significant differences according to the delay between MG onset and thymectomy (Fig. 2D) or between males and females (Fig. 2E). However, MGT patients with immunosuppressive treatments had higher AChR titers (Fig. 2F). We also observed a tendency for higher titers of anti-AChR antibodies for the B1, B2 and B3 thymoma subtypes relative to the A and AB thymoma subtypes (Fig. 2G).

3.2. Focus on germinal centers (GCs)

In nonthymomatous MG, the thymus is frequently characterized by B-cell infiltration and ectopic GC development [1]. In our database, 64.5% of the nonthymomatous MG patients displayed ectopic GCs. The percentage of GCs reached 78.8% in nonthymomatous AChR⁺ MG patients under 45 years old (data not shown). The presence of GCs was investigated in nonthymomatous MG by pathologists, while this was not always the case for MGT or TOMA patients. Nevertheless, in our database, for some patients with a thymoma, the presence of GCs was screened in the adjacent tissue of thymoma biopsies. Surprisingly, we observed that 58.8% of the MGT patients displayed ectopic GCs. In contrast, only 15.6% of TOMA patients were characterized by the presence of ectopic GCs (Table 1 and Fig. 3A). Knowing the impact of

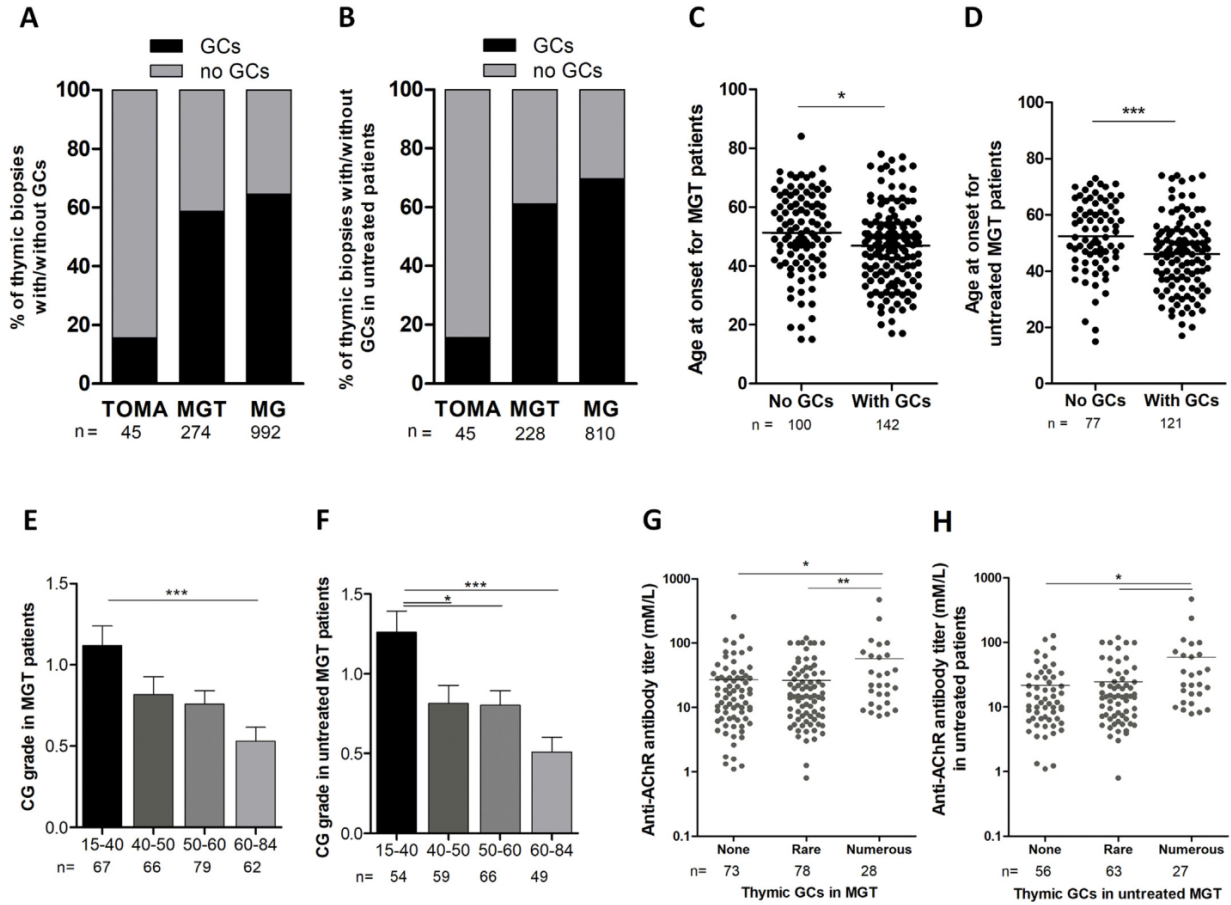


Fig. 3. Analyses of thymic GCs. (A–B) Percentages of patients with or without thymic GCs. (C–D) Age at MG onset according to the presence or not of thymic GCs in all MGT patients. (E–F) MGT patients were divided in four subgroups according to the age ≤ 40 , 40–50, 50–60 and ≥ 60 to analyze the degree of thymic hyperplasia that was graded as follows: no GC = 0; few GCs = 1; many GCs = 2; numerous GCs = 3. The data correspond to mean values \pm SEM. (G–H) Anti-AChR titers in 179 MGT patients (E) and 144 MGT patients without immunosuppressive treatments (F) according to the proportion of thymic GCs. (A, C, E, F) All patients and (B, D, F, G) patients without immunosuppressive treatments. p-values were assessed using the one-way ANOVA with Bonferroni post hoc tests (C–H) and p-values are indicated if $p < 0.05$ as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

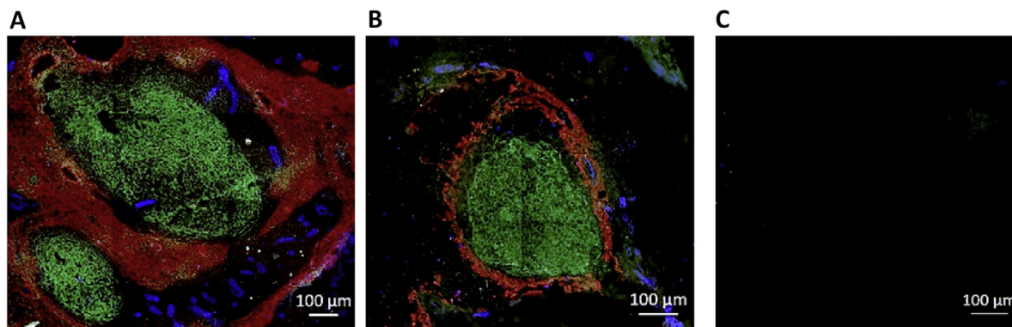


Fig. 4. Immunohistochemistry for thymic GCs in EOMG and MGT patients. (A–B) Immunofluorescence staining of representative thymic sections from EOMG (A) and MGT (B) patients with an anti-CD21 antibody to detect GCs (in green), an anti-PNAD for HEVs (in bleu) and an anti-cytokeratin for thymic epithelial cells (in red). Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope. (C) Negative control of the staining on a serial thymic section of the EOMG patient showed in A. Images are composed of six mosaic pictures. Scale bars = 100 μ m (for interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

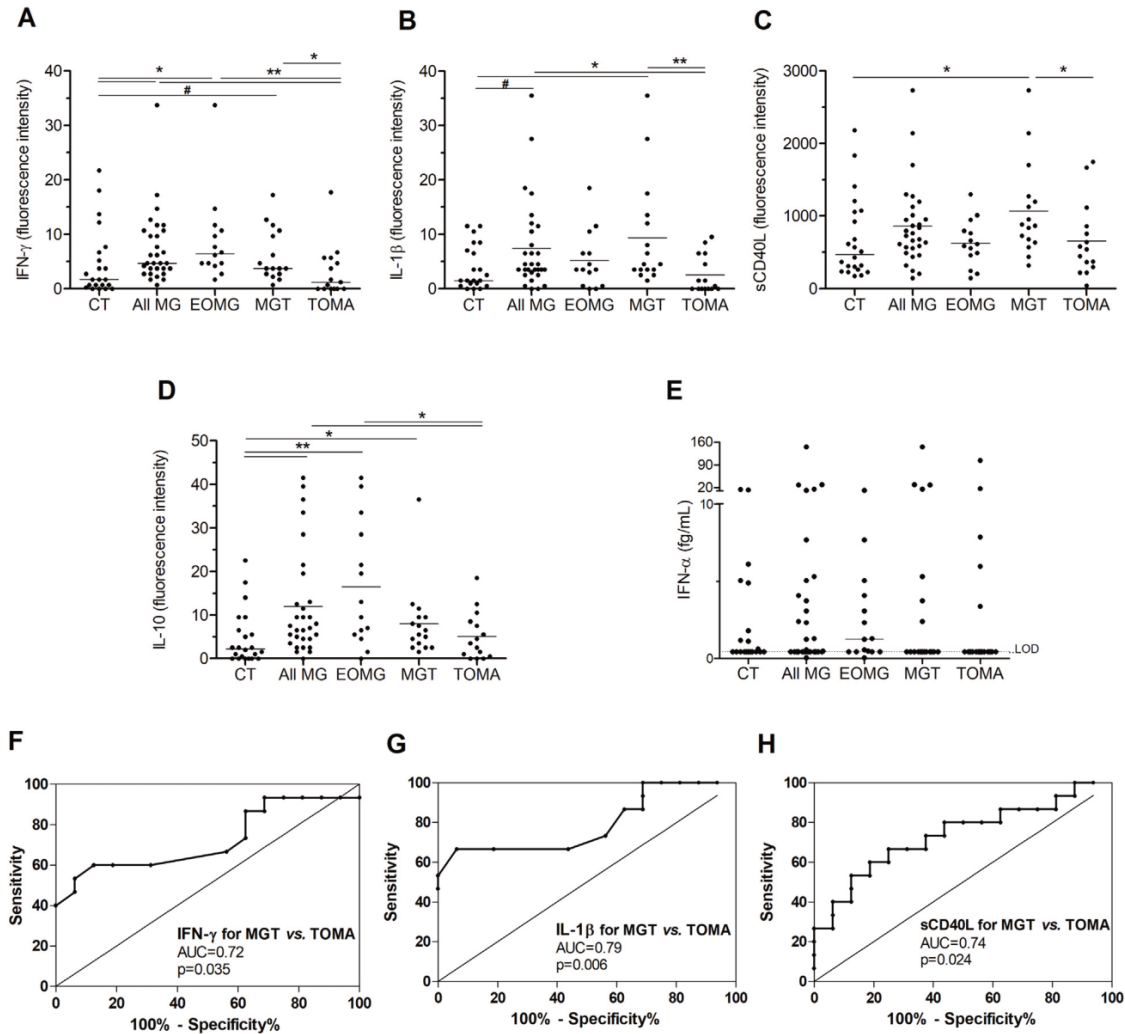


Fig. 5. Serum levels of cytokines in different groups of patients. (A–D) Multiplex analyses for IFN- γ (A), IL-10 (B), IL-1 β (C) and sCD40L (D) in control (CT, n = 22), EOMG (n = 14), MGT (n = 16) and TOMA (n = 15) donors. The “all MG” subgroup (n = 30) corresponds to EOMG and MGT patients altogether. Cytokine expression levels were analyzed using the fluorescence signal (minus the fluorescence background). (E) Simoa analysis for IFN- α subtypes in control (CT, n = 20), EOMG (n = 15), MGT (n = 19) and TOMA (n = 20) donors. The “all MG” subgroup (n = 34) corresponds to EOMG and MGT patients altogether. (A–E) p-values were assessed using a Kruskal-Wallis test with Dunn’s multiple comparisons and p-values are indicated as follows: (#) p < 0.1, (*) p < 0.05, (**) p < 0.01. (F–H) Analyses of the sensitivity and specificity of IFN- γ (F), IL-1 β (G) and sCD40L (H) as markers to discriminate MGT from TOMA patients. The ROC curves were computed using the expression level of the cytokines in MGT and TOMA patients.

cells are known to favor GC development by interacting with GC-B cells. They demonstrated that MGT patients have a higher percentage of Tfh cells than TOMA patients or controls [25]. In addition, Cavalcante et al. demonstrated an increased proportion of B cells and of the B-cell chemokine CXCL13 in the thymuses of MGT patients relative to TOMA patients or controls [26]. Here, by immunohistochemistry, we confirmed the presence of GCs in the adjacent thymic tissue but not in the thymomas of MGT patients. In particular, we showed the development of HEVs all around these GCs, exactly as in the thymus of EOMG patients [27]. In secondary lymphoid organs and in chronically inflamed tissues, such as the MG thymus, lymphocyte homing is directed through HEVs, a specialized endothelium bearing on its luminal surface diverse chemokines and expressing high levels of PNA α carbohydrate ligands [27,28]. In MG patients, thymic HEVs expressed the chemokines CXCL12 [27] and CXCL17 [29]. It would be of interest to analyze chemokines that are expressed on HEVs in MGT thymuses. Regardless,

the development of these specific endothelial vessels suggests the abnormal recruitment of peripheral cells in the thymuses of MGT patients, as observed for EOMG patients.

Ectopic GCs often develop at sites of inflammation where they influence the course of infection, autoimmune disease, transplant rejection and cancer. What is the exact role of thymic GCs in the thymuses of MGT patients?

In some cancers, as for colorectal carcinoma, the presence of GCs is associated with better patient survival, as GCs are involved in local and systemic antitumor responses [30]. An elevated number of GCs also correlates with the long-term survival of patients with non-small-cell lung cancer [31,32]. Consequently, thymic GCs could be protective against the tumor itself and favor thymoma patient survival. However, it is difficult to draw conclusions, as thymectomy is part of the treatment, and it is not possible to relate patient survival with the presence of thymic GCs in the long term.

Ectopic GCs in various tumors can participate in the immune response against tumor-associated antigens or self-antigens. Here, we observed that the number of GCs is correlated with higher titers of anti-AChR antibodies, as also observed for EOMG [33]. In addition, MGT patients are known to display various autoantibodies associated with other autoimmune diseases but also autoantibodies against striated muscle antigens or cytokines [34,35]. Consequently, thymic GCs could have a negative role, favoring autoimmunity in particular.

4.3. Elevated cytokine levels associated with MG development in thymoma

We searched for blood biomarkers that could characterize MGT patients and define TOMA patients at risk of developing MG. As high levels of IFN- γ subtypes are detected in the thymuses of MGT and not of TOMA patients [36], we used ultrasensitive Simoa technology to measure all IFN- α subtypes [9]. However, we did not detect any increased levels of IFN- α in MGT or TOMA patients, and we even observed that serum samples from MGT and TOMA patients were frequently below the LOD relative to controls and EOMG patients. MGT patients as well as TOMA patients can display anti-IFN- α autoantibodies [34], which may potentially explain our results and requires further study. As for IFN- β , which plays a central role in thymic changes in EOMG [37], we also analyzed serum levels by ELISA in controls and MGT patients but did not observe any variation (data not shown). Similarly, using Bio-Plex technology, Uzawa et al. did not observe serum variation for IFN- β in controls, EOMG, late-onset MG and MGT patients [38].

With a multiplex approach, we found 4 cytokines at higher levels in the sera of MGT patients. IFN- γ and IL-10 were upregulated in all MG patients, both MGT and EOMG patients, as specific markers for MG. IL-1 β and sCD40L were only detected significantly at higher levels in sera of the MGT group. Data analysis using ROC curves demonstrated that only IFN- γ , IL-1 β and sCD40L could discriminate MGT from TOMA patients.

Fang et al. also observed increased serum levels of IFN- γ in MG patients [39]. IFN- γ has pleiotropic effects, and a role in mediating autoimmune diseases has been previously suggested [40]. IFN- γ is important in inducing B-cell proliferation and differentiation, consequently favoring anti-AChR antibody production. IFN- γ is also required to induce experimental MG [41]. Elevated serum levels of IFN- γ could thus be a good predictive biomarker in thymoma patients susceptible to developing MG symptoms.

In 2014, Uzawa et al. measured the expression of 27 cytokines/chemokines in MGT and MG patients. They also described the significant upregulation of IL-1 β in MGT patients relative to controls [42]. IL-1 β is a proinflammatory cytokine that drives Th1 and Th17 cell differentiation [43] and favors autoimmunity. IL-1 β knockout mice are also resistant to the induction of an experimental autoimmune MG model [44]. The role of higher levels of IL-1 β in MGT is difficult to decipher as this cytokine can also be involved in tumor induction [45].

Of interest, our study is the first to show elevated levels of sCD40L in MGT patients. CD40 and CD40L are expressed on a wide variety of cells. In the circulation, sCD40L mainly derives from activated T cells and platelets. It corresponds to a cleaved form of the membrane CD40 protein but retains considerable biological activity and the ability to bind to the CD40 receptor. Elevated sCD40L levels are observed in pathological conditions, such as inflammation, neoplasia and autoimmune diseases [46]. It was also demonstrated that sCD40L increases serum IgG levels and GC formation in mice [47]. In an experimental model of MG, the use of anti-CD40L has been shown to suppress ongoing chronic MG [48]. The role of sCD40L thus seems complex and difficult to understand in MGT patients.

Altogether, these observations showed elevated levels of IFN- γ , IL-1 β and sCD40L in MGT patients. The levels of these cytokines should be analyzed prospectively in TOMA patients to correlate these cytokines

with the emergence of MG symptoms.

5. Conclusion

Our retrospective analysis of patients from our database, together with a review of the literature, lead us to propose a list of clinical parameters for thymoma patients at risk of developing MG symptoms after thymectomy. We clearly demonstrated that ectopic thymic GCs were associated with MG in thymoma patients and should be systematically searched by pathologists. From our analyses, a risk score could be established with the following parameters: 1) detection of anti-AChR antibodies before surgery, 2) B2 or B1 thymoma subtypes, 3) presence of ectopic thymic GCs, 4) local invasiveness of thymoma, 5) women before 50 years old, and 6) high serum levels of IFN- γ , IL-1 β and sCD40L. Now, a prospective study would be of interest to confirm the validity of these parameters. Early identification of patients at risk of developing MG is very important for specific follow-up after surgery. In addition, the benefits of early immunosuppressive treatment of these patients to prevent MG symptoms and/or to decrease their intensity should be discussed, knowing that this form of MG is more severe with bulbar and respiratory symptoms leading to prolonged treatment despite thymectomy [49].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2019.102337>.

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Author contributions

CL, CP, O-MF and SM performed and analyzed experiments, FT collected samples and managed patient information in the database, VB and DD performed Simoa experiments, VM, M-RG, AM-L, MA and PV provided histopathological reports after thymectomy. EF, BE and AB provided blood samples. EF and DG provided thymic samples. SB-A established the database. CL, CP, AB and SB-A read and revised the manuscript. CL and RLP designed the study, analyzed the experiments and wrote the manuscript.

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Article 3
**Decreased expression of mir-29 family associated with autoimmune myasthenia
gravis**

RESEARCH

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Decreased expression of miR-29 family associated with autoimmune myasthenia gravis



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Abstract

Background: Myasthenia gravis (MG) is a rare autoimmune disease mainly mediated by autoantibodies against the acetylcholine receptor (AChR) at the neuromuscular junction. The thymus is the effector organ, and its removal alleviates the symptoms of the disease. In the early-onset form of MG, the thymus displays functional and morphological abnormalities such as B cell infiltration leading to follicular hyperplasia, and the production of AChR antibodies. Type-I interferon (IFN-I), especially IFN- β , is the orchestrator of thymic changes observed in MG. As Dicer and miR-29 subtypes play a role in modulating the IFN-I signaling in mouse thymus, we investigated their expression in MG thymus.

Methods: The expression of DICER and miR-29 subtypes were thoroughly investigated by RT-PCR in human control and MG thymuses, and in thymic epithelial cells (TECs). Using miR-29a/b-1-deficient mice, with lower miR-29a/b-1 expression, we investigated their susceptibility to experimental autoimmune MG (EAMG) as compared to wild-type mice.

Results: DICER mRNA and all miR-29 subtypes were down-regulated in the thymus of MG patients and DICER expression was correlated with the lower expression of miR-29a-3p. A decreased expression of miR-29 subtypes was similarly observed in MG TECs; a decrease also induced in TECs upon IFN- β treatment. We demonstrated that miR-29a/b-1-deficient mice were more susceptible to EAMG without higher levels of anti-AChR IgG subtypes. In the thymus, if no B cell infiltration was observed, an increased expression of *Irf- β* associated with *Baff* expression and the differentiation of Th17 cells associated with increased expression of *Il-6*, *Il-17a* and *Il-21* and decreased *Tgf- β 1* mRNA were demonstrated in miR-29a/b-1-deficient EAMG mice.

Conclusions: It is not clear if the decreased expression of miR-29 subtypes in human MG is a consequence or a causative factor of thymic inflammation. However, our results from the EAMG mouse model indicated that a reduction in miR-29a/b1 may contribute to the pathophysiological process involved in MG by favoring the increased expression of IFN- β and the emergence of pro-inflammatory Th17 cells.

Keywords: microRNAs, DICER, Thymus, Thymic epithelial cells, Experimental autoimmune myasthenia gravis, Interferon- β , Th17 cells, BAFF

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Introduction

MG is a neuromuscular disease characterized by invalidating muscle weaknesses. It is caused by autoantibodies targeting components of the neuromuscular junction, such as the acetylcholine receptor (AChR) [1]. In early-onset MG, the thymus is the effector organ and its removal alleviates the symptoms of the disease. The MG thymus displays functional and morphological abnormalities characterized by abnormal B cell infiltration leading to follicular hyperplasia and the production of anti-AChR antibodies [1]. These data indicate that disordered thymic processes underlie MG; however, the molecular regulators of this dysfunction remain to be elucidated. A thymic overexpression of interferon (IFN)- β and IFN-I-induced genes is observed in MG, even long after disease onset, and IFN- β seems to be the main orchestrator of thymic changes [2–4]. Besides, a causative role of IFN-I is supported by a mouse model where injections of Poly(I:C) induce thymic changes that lead to an anti-AChR response [3, 4].

Papadopoulou et al. demonstrated that miRNAs are essential to protect thymic architecture. Using conditional knock-out mice for Dicer in thymic epithelial cells (TECs, *Foxn1^{Cre} Dicer^{f/f}*), they observed a premature involution and the appearance of epithelial voids with dense foci of B cells. Besides, Dicer-deficient mice are hypersensitive to Poly(I:C) in line with increased expression of *Ifnar1* in TECs. This latter effect is mediated by the miR-29a/b-1 cluster, as miR-29a targets *Ifnar1* and increases the sensitivity to Poly(I:C) due to an increased expression of *Ifnar1* in TECs [5].

These observations suggest a link between miR-29a and the IFN-I signature observed in MG thymus. Consequently, we investigated the expression of DICER and

miR-29 subtypes in the thymus of MG patients and investigated the sensitivity of miR-29a/b-1-deficient mice to experimental autoimmune MG (EAMG).

Methods

Human thymic samples

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 at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France). The degree of thymic follicular hyperplasia in MG patients was assessed by pathologists (high degree of hyperplasia (MH patients ($n = 6$)) with 3 or more germinal centers per section vs. low degree of hyperplasia (ML patients ($n = 6$)) with 2 or less germinal centers per section). 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.

Primary human TECs were cultured from infant thymus collected from donors undergoing cardiovascular surgery, and correspond mainly to medullary TECs as previously described [6]. TECs were seeded (1.4×10^5 cells/cm²) in RPMI-5% horse serum for 24 hours. Next, TECs were treated with IFN- β 1000 UI/ml (11415, R&D Systems, Lille, France) in RPMI-0.5% horse serum for 24 h.

Animals

Thymic miR-29a/b-1-deficient mice on the B6 background were designed as previously described [5]. They

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

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

(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)

were decontaminated upon arrival by embryo transfer in C57BL/6j mice purchased at Janvier Labs (Saint-Berthevin, France). Afterward, mice were bred and host 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).

RT-PCR for miRNAs on human thymic biopsies

Total RNA was extracted from human 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).

miRNAs were retro-transcribed from total RNA using the TaqMan MicroRNA Reverse Transcription Kit and following the Custom RT Pool protocol (ThermoFisher Scientific, Villebon-sur-Yvette, France). Briefly, 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 follows: 1 cycle of polymerase activation and denaturation at 95 °C for 10 min, 45 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. miRNA expression was normalized to 28S. Primers used for qPCR are listed in Table 2.

RT-PCR for mRNAs

Total RNA from human thymus was obtained as described above. Total RNA from mouse thymus or from TEC cultures were extracted in TRIzol (ThermoFisher Scientific) using the FastPrep FP120 instrument (Qbiogen, Illkirch, France) for thymic biopsies. RNA (1 µg) was reverse-transcribed for 1 h at 42 °C using AMV (Ref 10109118001, Roche Life Science, Meylan, France) with oligo-dT (ThermoFisher Scientific). PCR reactions were carried out with the LightCycler 480 SYBR Green Master Mix on the LightCycler® 480 System (Roche). All samples were normalized to 28S or GAPDH. The primer

sequences (Eurogentec, Angers, France) are listed in Table 3.

Experimental autoimmune myasthenia gravis (EAMG)

Six to 8-week-old C57BL/6 male and female mice were used as inbred miR-29 a/b-1 heterozygous (HET), WT siblings on the C57BL/6 background, and additional C57BL/6j WT mice from Janvier Labs. Male and female mice were combined in the analyses, with no sex-based differences observable in the EAMG model [7]. The extraction of Torpedo Californica AChR (T-AChR) was led as previously described [8]. T-AChR was emulsified with an equal volume of Complete Freund's adjuvant (CFA; F5881, ThermoFisher Scientific) 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 4 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 graded from 0 to 9 by taking into account the weight evolution, muscle strength, and the inverted grid test. Each of these measures was graded on 3, as described by Weiss et al. with minor modifications as follows [8]. The grip test measurements were done after a 5-min run in a treadmill. Grip test values were normalized on the weight of the animals since experiments were performed on female and male mice. For each mouse, the weight and grip test measures were compared to those obtained before the immunization, and a percentage representing the loss/gain of weight or of muscle strength along the experiment was determined. The grading on 3 was then made as detailed by Weiss et al. [8]. 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 signs of abnormal behavior. Mice were considered sick when they reached a global clinical score of 2. Mice reaching a global clinical score of 9 were euthanized.

Table 2 List of TaqMan microRNA assays used for custom RT pool and qPCR on miRNAs

Assay ID	miRNA name	miRBase ID	Sequence
002112	hsa-miR-29a-3p	MIMAT0000086	UAGCACCAUCUGAAAUCGGUUA
002447	hsa-miR-29a-5p	MIMAT0004503	ACUGAUUUUUUGGUGUUCAG
000413	hsa-miR-29b-3p	MIMAT0000100	UAGCACCAUUUGAAAUCAGUGUU
002165	hsa-miR-29b-1-5p	MIMAT0004514	GCUGGUUUCAUUUGGUGGUUAGA
002166	hsa-miR-29b-2-5p	MIMAT0004515	CUGGUUUCACAUUGGUGGUUAG
000587	hsa-miR-29c-3p	MIMAT0000681	UAGCACCAUUUGAAAUCGGUUA
001818	hsa-miR-29c-5p	MIMAT0004673	UGACCGAUUUCUCCUGGUGUUC

Table 3 List of primers used for RT-PCR on mRNAs

	Gene name	Sens primer	Antisens primer
Human primers	<i>28S</i>	GGTAGGGACAGTGGGAATCT	CGGGTAAACGGCGGGAGTAA
	<i>CD19</i>	TACTATGGCACTGGCTGCTG	CACGTTCCCGTACTGGTTCT
	<i>DICER</i>	TGCTGAACTGCAACTGACC	CAGGGTCCCAAGTACCAA
	<i>GAPDH</i>	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG
	<i>IFNAR1</i>	CCTCTGTGAGCCTAAGTGC	AAGGGCTACCTCAGTGTT
Mouse primers	<i>Baff</i>	TTCCATGGCTTCTCAGCTTT	GGAATTGTTGGCAGTGTTT
	<i>Ccl21</i>	CCCTGGACCAAGGCAGT	AGGCTTAGAGTGCTCCGGG
	<i>Cd19</i>	CCCTCACCTCGAGTTTCTG	TAGGTTACAGGTCCCAAGG
	<i>Cxcl13</i>	TGAGGCTCAGCACAGCAA	ATGGGCTCCAGAATAACCG
	<i>Cxcr5</i>	ATGGCCTTAATGTGCTGTC	CTTCTGGAACCTGCCCTCAG
	<i>Gapdh</i>	AACCTTGGCATTGTGGAAGG	ACACATTGGGGTGAAGAAC
	<i>Ifn-β</i>	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTCA
	<i>Ifn-α2</i>	TCTGTGCTTCTCCTGTGATG	TTGAGCCTTCTGGATCTGCT
	<i>Ifnar1</i>	CAAGTGTGCTGGCTTGTTC	AGAGAAGTCCGAGGCCATCT
	<i>Ifnar2</i>	ACATGGGTCTGGCTCAAAG	GGCAGAGAAAGGGTGTCTCT
	<i>Ifn-γ</i>	CAGCAACAGCAAGGCGAAA	GCTGGATTCCGGCAACAG
	<i>Il-4</i>	GGTCTCAACCCCGCTAGT	GCCGATGATCTCTCAAGTGAT
	<i>Il-6</i>	AGTTGCTTCTGGGACTGA	TCCACGATTCCAGAGAAC
	<i>Il-10</i>	GCTCTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
	<i>Il-17a</i>	TTAACTCCCTTGGCGAAAA	CTTCCCTCCGATTGACAC
	<i>Il-21</i>	GGACCCTGTCTGTCTGGTAG	TGTGGAGCTGATAGAAGTTCAGG
	<i>Il-23</i>	CCGTTCCAAGATCCTTCGAA	GACCCGGGCTGCTATGG
	<i>Tgf-β1</i>	CAAGGGCTACCATGCCAACT	CCGGGTGTGTTGGTTGTAGA

ELISA for anti-AChR antibodies

ELISA experiments were carried out on serum samples collected at sacrifice (day 43). 96-well plates were coated with 0.5 µg/mL of T-AChR 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. Samples were diluted in PBS 0.2% BSA (1:100 000) and incubated at 37 °C for 1 h and 30 min. One hundred microliters of biotinylated rabbit anti-mouse IgG (1/10000, E0413, Dako, Courtaboeuf, France) or biotinylated anti-mouse IgG subtypes were added for 1 h and 30 min at 37 °C (anti-IgG2b 1/250 (553393, BD Biosciences, Le Pont de Claix, France); anti-IgG2c (1/5000, SA5-10235, ThermoFisher Scientific) or anti IgG1 (1/100; 553441, BD Biosciences). Samples were incubated 30 min with 100 µL of streptavidin–horseradish peroxidase (diluted at 1:10 000) (S911, ThermoFisher Scientific). Tetramethylbenzidine was used for color development, and the optical density at 450 nm was measured with the SPARK 10 M microplate reader (TECAN Life Sciences, Grödig, Austria). Between each step, wells were washed 4 times with 200 µl of PBS 0.05% Tween 20.

Detection of relative affinity of anti-AChR IgG antibodies

The relative affinity of anti-AChR IgG antibodies was determined based on the above ELISA method using a potassium thiocyanate (KSCN) elution step [9]. Increasing concentrations of KSCN in 0.1 M PBS (pH 6.0) for 15 min at room temperature before the biotinylated rabbit anti-mouse IgG. KSCN concentrations ranging from 1 to 8 M were tested. As anti-AChR antibodies were totally removed at higher KSCN concentrations than 4 M of KSCN. Concentrations below 4 M of KSCN were used for linear regression in GraphPad and the relative affinity corresponds to the molarity of KSCN resulting in 50% of the absorbance obtained in the absence of KSCN.

Immunohistochemistry

Cryosections of thymic samples (7 µm) were fixed in ice-cold acetone for 20 min and unspecific binding sites blocked with 2% BSA. Sections were stained for medullary thymic epithelial cells with a Keratin 5 polyclonal antibody (Biolegend, Ozyme, Saint-Cyr-L'école, France) and a donkey anti-rabbit IgG Alexa-Fluor-488 (ThermoFisher

Scientific), while B cells were detected with a B220 biotinylated antibody (clone RA3-6B2, BD Biosciences) and streptavidin Alexa-Fluor-594 (S11227, ThermoFisher Scientific). Images were acquired with a ZeissAxio Observer Z1 Inverted Microscope. The number of B cells was counted in 6–8 fields representative of each thymic section.

Statistical analysis

For 2-by-2 comparisons, the non-parametric Mann-Whitney test was applied as specified in figure legends. To analyze the mouse susceptibility to EAMG, the comparisons among the different mouse groups in kinetic were performed using a two-way ANOVA with Bonferroni adjustment for multiple comparisons. Correlation analyses were performed using Spearman's correlation coefficient for non-Gaussian distributed variables, with a $p < 0.05$ considered significant.

Results

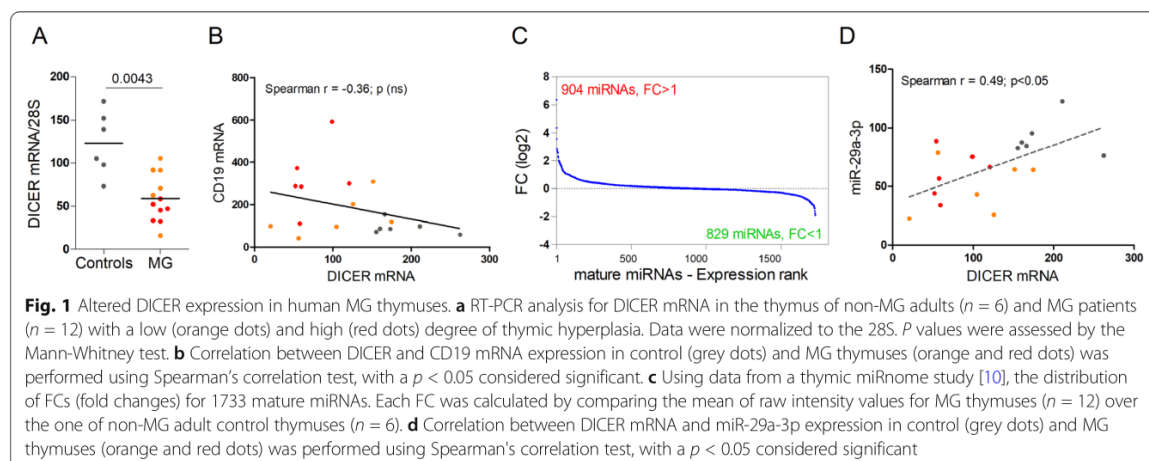
Decreased expression of DICER correlates with reduced miR-29a-3p expression in MG patients

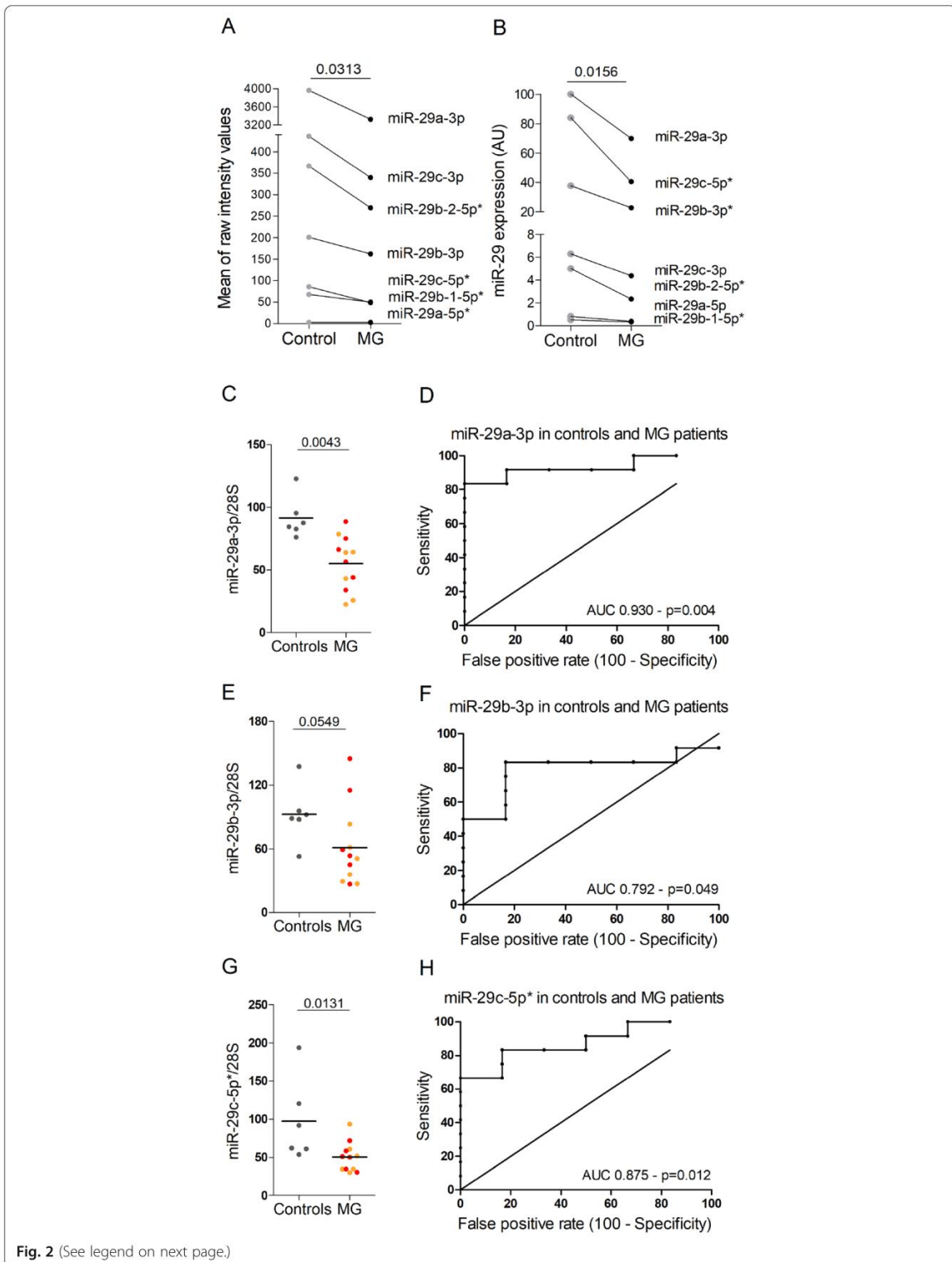
To investigate the putative role of the DICER/miR-29/IFN-I axis in MG, we analyzed the expression of DICER mRNA, a key enzyme in miRNA biogenesis. We observed a 4.6-fold decrease of expression in the thymus of MG patients, compared to healthy control thymus (Fig. 1a). Even if the DICER mRNA expression seemed lower in patients with a high degree of follicular hyperplasia (Fig. 1a), this was independent of the abnormal presence of B cells in the thymus. Indeed, no correlation between CD19 and DICER mRNA expression was observed (Fig. 1b). To assess the impact of reduced DICER expression on the global miRNA profile, we analyzed published miRnome data obtained from the same thymic biopsies [10]. Analyzing the expression of 1733 mature thymic miRNAs in MG patients as compared to controls, we observed almost an equal repartition of up- and down-miRNAs, with 904 and

829 miRNAs with a fold change (FC) higher and lower than 1, respectively (Fig. 1c). Despite no systematic impact of reduced DICER expression on the global expression of thymic miRNAs, we observed a significant correlation between DICER mRNA and miR-29a-3p expression (Fig. 1d). As Papadopoulou et al. demonstrated that DICER deletion in TECs increased sensitivity to IFN-I signaling and that miR-29a was the best candidate to mediate this effect [5, 1]. This association of reduced DICER and miR-29a expression in the MG thymus warranted further investigation as a potential causative link.

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

The miR-29 subtypes are expressed by two genomic clusters: the miR-29a cluster (miR-29a/b-1 on chromosome 1 in human and mice) and the miR-29c cluster (miR-29b-2/c on chromosome 7 in human and chromosome 1 in mice) [11]. Using miRnome data previously published [10], we observed a significant decrease expression of all the members of the miR-29 family (miR-29a-3p, miR-29c-3p, miR-29b-2-5p*, miR-29b-3p, miR-29c-5p*, miR-29b-1-5p* and miR-29a-5p*) in MG thymuses compared to control thymuses (Fig. 2a). To validate these observations, we analyzed by RT-PCR the expression of all miR-29 subtypes. We observed that miR-29a-3p was the most strongly expressed miR-29 thymic subtype (Fig. 2a, b), as consistent with the data from the mouse thymus [5]. We confirmed that all miR-29 miRNAs were significantly downregulated in MG thymuses except the very low expressed miR-29b-1-5p* (Fig. 2b). Detailed results are showed for the three most highly expressed miRNAs, miR-29a-3p, miR-29b-3p, and miR-29c-5p* (Figs. 2c, e, g). The decreases were observed in all MG thymuses independent of the degree of thymic hyperplasia. In addition, ROC curve analyses demonstrated the high sensitivity and





(See figure on previous page.)

Fig. 2 Decreased expression of miR-29 subtypes in human MG thymuses. **a** miR-29 subtypes expression was assessed using data from a thymic miRNome study [10]. Each point corresponds to the mean of raw intensity values for 6 non-MG adult control thymuses and 12 MG thymuses. *p* value was assessed by the Wilcoxon test. **b–h** miR-29 subtypes expression was assessed by RT-PCR in 6 non-MG adult control thymuses and 12 MG thymuses. **b** Each point corresponds to the mean values for the controls and MG thymuses. *p* values were assessed by the Wilcoxon test. **c, e, g** Detailed expression of miR-29a-3p, miR-29b-3p, and miR-29c-5p in control and MG thymuses with a low (orange dots) and high (red dots) degree of thymic hyperplasia. Data were normalized to the 28S. *p* values were assessed by the Mann-Whitney test. **d, f, h** Analyses of the sensitivity and specificity with ROC curves for miR-29a-3p, miR-29b-3p, and miR-29c-5p* in control and MG thymuses

specificity for these three miR-29 subtypes for discriminating MG patients from controls (Fig. 2d, f, h).

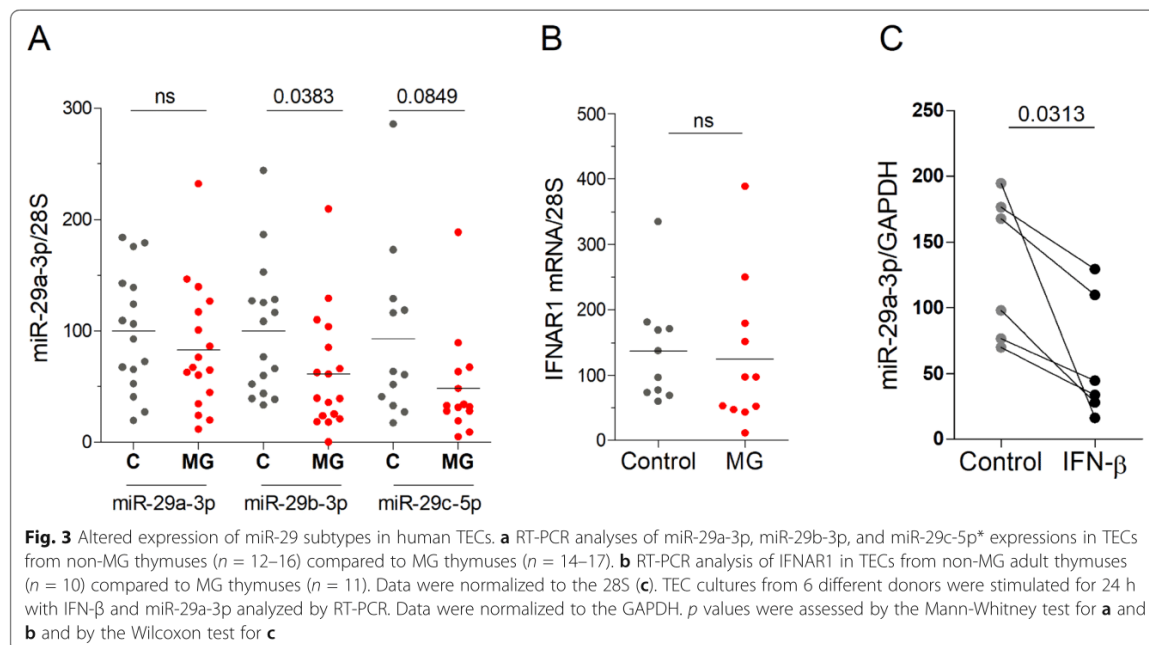
These data demonstrated that miR-29 subtypes were all downregulated in the thymus of MG patients, potentially leading to a higher expression of genes targeted by miR-29 miRNAs.

miR-29 subtypes are downregulated in TECs from MG patients

To determine the cellular source of miR-29 dysregulation in the MG thymus, we analyzed the level of expression of the three main thymic miR-29 subtypes in TECs derived from thymic explant cultures. We observed decreased expression or downwards trends for each of the three miR-29 subtypes tested (Fig. 3a). We also analyzed miR-29a-3p, miR-29b-3p, and miR-29c-5p* expression in thymocytes/lymphocytes freshly extracted from 5 MG and 5 control thymuses. miR-29a-3p and miR-29b-3p were expressed at 4–7-fold lower levels in thymocytes versus TECs from control donors, and miR-29c-5p* was borderline undetectable in thymocytes. No decrease

expression of miR-29 subtypes was observed in thymocytes from MG patients compared to non-MG controls (data not shown), further supporting that the decreased expression in TECs could reflect what was observed at the whole thymus level. Nevertheless, the decrease in TECs was less significant than in the whole MG thymus probably as TECs were analyzed after 7 days in culture conditions that are different than the inflammatory thymic MG context.

Despite the decrease expression of the three miR-29 subtypes, no increased expression for IFNAR1 was observed (Fig. 3b). This suggests that there might be a threshold level for miR-29 to decrease IFNAR1 mRNA expression in TECs. In addition, human IFNAR1 is not listed as targeted by miR-29a according to the usual sequence matching databases (miRanda, TargetScan, and DIANA microT), and thus species differences clearly exist. A link between miR-29 and the IFN-I pathway was assessed through the exposure of TECs from healthy donors to IFN- β , as IFN- β is known to be overexpressed in the thymus of MG patients [3]. Through analyzing



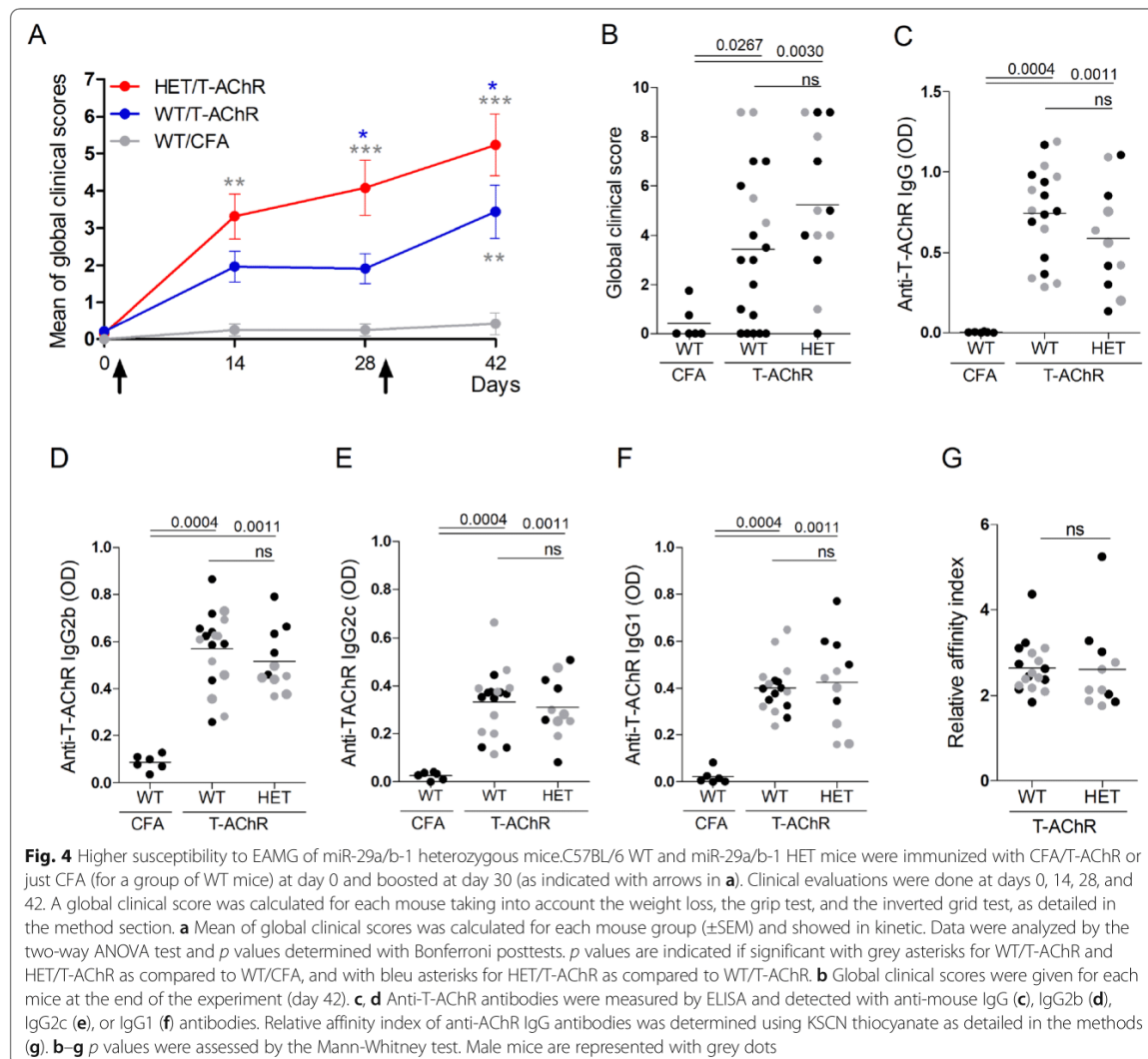
the effect of IFN- β on miR-29a-3p, the most highly expressed miR-29 subtype, we observed that IFN- β induced a decreased expression of miR-29a-3p (Fig. 3c). The effect of IFN- β on miR-29a-3p expression is independent of DICER as IFN- β did not affect DICER mRNA expression in TECs (data not shown). Together, these data indicate that the down-regulation of miR-29 subtypes in the thymus of MG patients could be due to a lower expression of these miRNAs in TECs, potentially mediated in response to high levels of IFN- β exposure in the MG thymus.

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

The observation of reduced DICER and miR-29a expression in MG patient thymuses suggests a potential

causative pathway for disease. Based on the known biology of miR-29a, the reduced expression could elevate response to IFN-I, escalating the disease process. Conversely, the identified expression changes in patients could be correlative, rather than causative. In order to test a potential causative link, we turned to the mouse model. Mice heterozygous for the deletion in the miR-29a/b-1 cluster (HET mice) mimic the 2-fold expression decrease observed in MG patients [5]. We, therefore, sought to compare the response of HET and wild-type (WT) control mice to the induction of MG, using the EAMG model.

Using a global clinical score, we observed that miR-29a/b-1 HET mice were more susceptible to EAMG induction compared to WT mice. HET mice developed symptoms more rapidly than WT mice even before the



boost immunization (Fig. 4a). At the end of the experiment, 92% of HET mice were sick (with a global clinical score over 2) as compared to only 63% for the WT mice (Fig. 4b). We measured the serum levels of anti-T-AChR IgG and specifically anti-AChR IgG1, IgG2b, and IgG2c (IgG2 isotypes acting via the complement in C57BL/6 mice). All mice immunized with T-AChR developed anti-AChR antibodies that were not detected in CFA-injected control mice. However, we did not observe any differences between miR-29a/b-1 HET mice compared to WT mice (Figs. 4c–f). We also measured the levels of anti-mouse AChR IgG, IgG2b, and IgG2c by coating the ELISA plates with a mouse AChR peptide but did not detect any increased level of anti-mouse AChR antibodies in miR-29a/b-1 HET mice (data not shown). Consequently, the levels of anti-AChR antibodies did not explain the higher clinical score observed in miR-29a/b-1 HET mice. To characterize the properties of anti-AChR antibodies from WT as compared to miR-29a/b-1 HET mice, we measured the antibody affinity but did not detect any difference between the two mouse strains (Fig. 4g).

Upon induction of EAMG, HET mice exhibited strong thymic involution (Fig. 5a) consistent with published results. A decrease in cellularity is observed in the thymus of miR-29 KO mice from 9 weeks old but the proportion of double-positive, double-negative, CD4, or CD8 thymocytes are not affected [5]. Unlike the published work, however, we did not detect an increase in CD19⁺ B cells in the thymus of HET mice, by either immunohistochemistry (Figs. 5b, c) or by RT-PCR (Fig. 5d). In addition, no increases were detected for *Cxcl13* or *Ccl21* mRNAs (Figs. 5e, f), two chemokines involved in B cell recruitment in MG thymus, or for *Cxcr5* mRNA (Fig. 5g), *Cxcl13* receptor essential for B recruitment [12, 13]. *Baff* mRNA expression was significantly upregulated in the thymus of miR-29a/b-1 HET mice compared to WT and CFA mice (Fig. 5h).

Regarding the IFN-I signature, no increased expression in *Ifnar1* or *Ifnar2* mRNAs was observed in miR-29a/b-1 HET mice (Figs. 5i–j). However, in the thymus of miR-29a/b-1 HET mice, *Ifn-β* mRNA was significantly increased compared to WT mice (Fig. 5k) and *Ifn-α2* mRNA was also slightly increased but only compared to CFA mice (Fig. 5l). Cytokines defining T cell subsets were also analyzed by RT-PCR: *Ifn-γ* (expressed by Th1 cells), *Il-4* (expressed by Th2 cells), *Il-10* and *Tgf-β1* (expressed by Treg cell), *Il-17a* and *Il-21* (expressed by Th17 cells), *Il-6*, *Il-23* (inducing Th17 differentiation). No changes were observed for *Ifn-γ*, *Il-4*, *Il-10* (Fig. 5m, o) and a decrease was observed for *Tgf-β1* (Fig. 5p) in miR-29a/b-1 HET compared to WT mice. However, we observed a clear increased expression of *Il-17a* and *Il-21* in miR-29a/b-1 HET mice as compared to WT mice (Fig. 5r, t, u) suggesting an

increase in Th17 cells. Accordingly, the expression of *Il-6* known to favor Th17 differentiation was also increased in the thymus of miR-29a/b-1 HET mice (Fig. 5r) but not that of *Il-23* (Fig. 5s).

Altogether, these results demonstrate that reduced miR-29 can have a direct pathological impact on the disease MG susceptibility. This is independent of thymic B cell infiltration but could be associated with a stronger Th17 signature.

Discussion

miRNAs are potent modulators of protein expression and are therefore involved in many physiological and pathophysiological processes. Specific miRNAs are already known to be involved in thymic pathogenesis associated with MG [10, 14–16]. Here, we investigated the implication of DICER and the miR-29 family in thymic changes in early-onset MG.

We observed a significant decreased expression of DICER in MG thymuses. Papadopoulou et al. showed the importance of Dicer in mouse thymic architecture by deleting Dicer specifically in TECs [5]. They observed a premature thymic involution, the formation of epithelial voids, and the presence of dense B cell foci. In early-onset MG, the thymus is characterized by increased presence of B cells and germinal center development [1]. However, here, we did not observe a correlation between DICER expression and the degree of follicular thymic hyperplasia or the expression of CD19 mRNA. The decreased expression of DICER in the thymus of MG patients did not seem as critical as the total deletion of Dicer in TECs in mice, as it did not lead to a global decreased expression of thymic miRNAs. However, DICER also possesses non-canonical, miRNA-independent roles [17]. DICER protects cells from cytotoxic accumulation of endogenous dsRNAs, such as Alu RNAs that can lead to abnormal activation of damage-associated molecular patterns (DAMPs) and subsequently sterile inflammation associated with IFN-I signaling [18]. Cufi et al. demonstrated that the injection of dsRNA to mice leads to thymic changes in link with IFN-I signaling and favors the development of a specific autoimmune reaction against AChR [3].

We observed a significant correlation between DICER expression and miR-29a in human MG thymuses. In light of the pathological nature of IFN-I in MG [3, 4], and the role of miR-29a in desensitizing the thymus to IFN-I [5], this suggests a putative pathological pathway. The proposed pathway between Dicer and IFN-I signaling in the mouse is via miR-29a/b-1 regulation of *Ifnar1* expression in TECs. Elevated *Ifnar1* in TECs would explain the increased sensitivity of miR-29a/b-1-deficient mice to pathogen-related signals, as demonstrated with the injection of Poly(I:C) [5]. Here, we observed that all

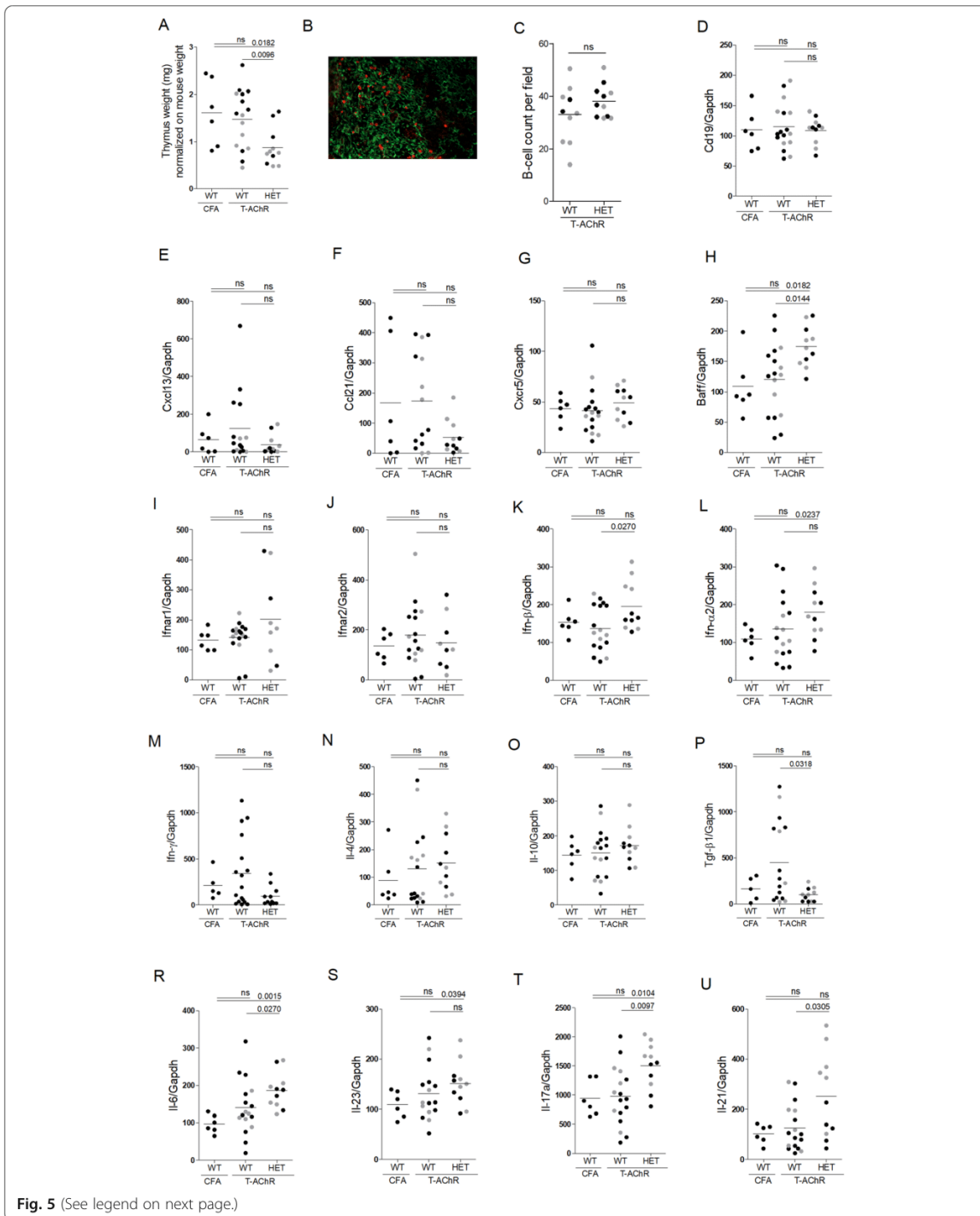


Fig. 5 (See legend on next page.)

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Fig. 5 Inflammatory thymic signature in miR-29a/b-1 heterozygous mice in the EAMG model C57BL/6 WT and miR-29a/b-1 HET mice were immunized with CFA/T-AChR or just CFA (for a group of WT mice) at day 0 and boosted at day 30. Mice were sacrificed at day 43. Male mice are represented with grey dots. **a** The weight of thymuses was normalized to mouse weight. **b** Representative picture of thymus labeling for cell counting. Thymic sections were stained for TECs with a Keratin 5 antibody (green) and for B cells with a B220 antibody (red). **c** The number of B cells was counted in 6–8 fields representative of thymic sections and each point corresponds to the mean for each mice. **d–u** RT-PCR analyses for *Cd19*, *Cxcl13*, *Ccl21*, *Cxcr5*, *Ifnar1*, *Ifnar2*, *Ifn-β*, *Ifn-α2*, *Il-6*, *Il-23*, *Il-17a*, *Il-21*, *Ifn-γ*, *Il-4*, *Il-10*, and *Tgf-β1* mRNA expression in mouse thymuses. Data were normalized on GAPDH. *p* values were assessed by the Mann-Whitney test

miR-29 subtypes were downregulated in the thymus of MG patients. This decreased expression was also observed in TEC cultures deriving from MG thymuses and could be in link with the inflammatory status of the MG thymus, as we observed a decreased expression of miR-29a in TECs upon IFN- β treatment. In multiple sclerosis patients, a downregulation of miR-29 family is also observed in peripheral blood mononuclear cells upon IFN- β treatment [19]. Besides, a decreased expression of miR-29a has also been observed in other inflammatory conditions, such as in fibroblasts in systemic sclerosis [20], in lung and plasma in chronic obstructive pulmonary disease [21], in renal tissues in IgA nephropathy [22], in peripheral blood T cells in Hashimoto's thyroiditis patients [23], and upon bacterial infection in IFN- γ producing cells [24].

As miR-29 subtypes were downregulated by about 50% in MG thymuses, we wondered if miR-29 HET mice were more susceptible to EAMG and displayed thymic changes as in the human disease. We demonstrated that miR-29 HET mice were more susceptible to EAMG according to the global clinical score. A decrease of thymic miR-29 miRNAs could favor the emergence of autoreactive T cells against α -AChR, as the absence of miR-29a/b-1 cluster selectively affects the Aire-dependent tissue-specific antigen expression in TECs [25]. Nevertheless, this enhanced susceptibility to EAMG does not seem due to a genetic increase in autoimmunity, as miR-29a/b-1 KO are protected in the collagen-induced arthritis (CIA) mouse model [26].

In our EAMG experiment, miR-29a/b-1 HET mice did not display higher anti-AChR antibody titers and the affinity of their anti-AChR antibodies was not altered either. Similarly, in MG patients, the severity of the disease is not correlated with the antibody titer [1], and it is correlated with the degree of thymic follicular hyperplasia [27]. However, we did not observe increased B cell infiltration and follicular hyperplasia in the thymus nor did we observe increased expression of chemokines susceptible to induce B cell recruitment. It should be noted that while the EAMG model with WT C57BL/6 mice leads to the production of anti-AChR antibodies and muscular symptoms as for MG patients, this model does not show any of the thymic abnormalities observed in the human disease [28]. In mice,

a strong combination of inflammation and CXCL13 overexpression seems mandatory to initiate thymic B cell recruitment [29, 8].

It was previously published that the regulation of *ifnar1* expression by miR-29a was a likely molecular mediator [5]. We did not observe an increased thymic expression of *Ifnar1* either because the decrease in miR-29a/b1 in heterozygous mice was not sufficient or because mRNA analyses were done in whole thymus and not in purified TECs. *Ifnar2* mRNA expression, that could also be targeted by miR-29a (www.targetscan.org), was not altered either. However, in our EAMG experiment, we showed the overexpression of thymic IFN- β mRNA in miR-29a/b1 HET as compared to WT mice. In WT C57BL/6 mice, a thymic increase in *Ifn-β* promotes the overexpression of chemokines such as CXCL13 and CCL21 that favor B cell recruitment [4]. It is possible that in heterozygous miR-29 mice the level of *Ifn-β* was not sufficient to trigger the expression of these chemokines and a subsequent B cell recruitment. It was nevertheless strongly associated with the overexpression of Baff, and the differentiation of Th17 associated with increased expression of *Il-6*, *Il-17a*, and *Il-21* mRNA. Baff expression increased in MG thymus [4]. Baff is known to induce the expansion of activated B cells and their survival but no increased number of B cells was detected in EAMG WT and miR-29 HET mice. Recent research works have also demonstrated that Baff can promote T cell activation, proliferation, and differentiation, in particular for Th17 cells [30]. Zhou et al. demonstrated that constitutive overexpression of Baff in transgenic mice promotes the generation of Th17 cells and aggravates experimental autoimmune encephalomyelitis (EAE) [31]. A decrease in *Tgf-β1* was also detected in the thymus of miR-29-deficient mice in the EAMG model. *Tgf-β1* plays an important role in controlling autoimmunity and abnormal inflammation by providing signals limiting immune activation and in particular suppressing Th17 cells expansion [32]. At low concentrations, *Tgf-β1* synergizes with *Il-6* and *Il-21* favoring Th17 cell differentiation [33]. In the human MG disease, a Th17 signature is also observed in the thymus [34]. This Th17 signature could explain that miR-29a/b1 HET were more severely affected in the EAMG model.

Conclusion

So far, most of thymic miRNAs described as dysregulated in early-onset are associated with GC development, such as miR-7, miR-24, miR-139, miR-143, miR-145, miR-146, miR-150, miR-452, miR-548, or thymic inflammation, such as miR-125b and miR-146 [16]. Here, we showed that miR-29a/b1 seems also link to thymic inflammation. In mouse, if a decrease of miR-29a/b1 can optimized IFN-I signaling by modulating *Ifnar1* mRNA expression in TECs [5], it could also lead to *Ifn-β* mRNA expression in the context of EAMG, in addition to other pro-inflammatory cytokines such as *Il-6*, *Il-17a*, and *Il-21*. In the human thymus, a decreased expression of miR-29a-3p was observed in MG patients and also in TECs upon IFN-β treatment. Yet, it is not clear if the decreased expression of miR-29 subtypes in MG is either a consequence or a causative factor of the thymic inflammation. Anyhow, our results indicated that a reduction in miR-29 subtypes may contribute to the pathophysiological process involved in MG by favoring the emergence of pro-inflammatory Th17 cells.

Abbreviations

AChR: Acetylcholine receptor; CFA: Complete Freund's adjuvant; HET: Heterozygous; IFN: Interferon; miRNA: MicroRNA; MG: Myasthenia gravis; TEC: Thymic epithelial cell; WT: Wild-type

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Authors' contributions

MAC, CAP, O-MF, and SM performed and analyzed the experiments. FT collected samples and provided patient information. EF and JG provided thymic biopsies. AL provided miR-29a/b-1-deficient mice. SB-A and AL read and revised the manuscript. MAC and RLP designed the study, analyzed the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author.

Ethics approval and consent to participate

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. The animal experimental protocols were approved according to European and French ethics agreements (n° 2569.01).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Abstract

Myasthenia gravis (MG) is a neuromuscular disease caused by autoantibodies against the acetylcholine receptor (AChR). Thymic abnormalities are associated with MG such as ectopic germinal centers with B cells producing anti-AChR antibodies. The MG thymus is also characterized by the overexpression of interferon (IFN)- β and IFN-stimulated genes. IFN- β plays a central role in MG, as it can induce the overexpression of α -AChR by thymic epithelial cells (TEC), of chemokines responsible for B cell infiltration, and of cytokines responsible for a T cell differentiation toward pro-inflammatory Th17, all favoring germinal center development leading to the autoimmune reaction against α -AChR.

IFN-I is classically overexpressed upon viral infection but so far, no specific viral signature has been found in MG patients. Actually, a new family of Mendelian diseases or some autoimmune diseases are now classified as Type-I interferonopathies. They are characterized by autoinflammation and a persistent IFN-I signature in the periphery.

My PhD aimed to understand the mechanisms causing chronic IFN-I production in the thymus of MG.

Initially, by analyzing serum and PBMC, I did not find any IFN-I signature in the periphery in MG patients. This underlines the fact that this signature is restricted to the thymus and that MG would be better defined as an organ-specific interferonopathy.

Next, my objectives were to understand the origin of thymic IFN in MG. IFN-I are produced upon pathogen infections but can also be associated with sterile inflammation due to defects in nucleic acid metabolism or a poor resolution of inflammation. I demonstrated that molecules mimicking endogenous nucleic acids can induce α -AChR expression by TEC via IFN- β release. Knowing that the thymus is the site of huge apoptotic processes for thymic selection and also in the context of acute thymic involution, we speculate that nucleic acids could come from apoptotic/necrotic thymocytes. To test this hypothesis, I co-cultured apoptotic/necrotic thymocytes with TEC and demonstrated an overexpression of IFN- β and α -AChR by TEC. Moreover, mice injected with dexamethasone that induces an acute thymic involution due to thymocyte apoptosis display an overexpression of IFN-I and α -AChR. *In vitro* and *in vivo* results agree with the potential implication of endogenous nucleic acids from necrotic thymocytes in the thymic IFN-I signature.

Apoptotic cells are normally engulfed by phagocytes, such as macrophages, in a process called efferocytosis. If not processed, apoptotic cells progress to a secondary necrosis stage and release their intracellular content. I demonstrated that thymic macrophages were decreased in MG as compared to normal thymus. We hypothesized that a defect in macrophages and clearance of apoptotic cells could lead to thymic inflammation and MG development. By inducing a depletion in macrophages in mice, I demonstrated that the thymus of mice overexpressed IFN-I and α -AChR. These results indicate that eNA from necrotic cells could be responsible for overexpression of IFN-I in the thymus of injected mice and perhaps in the MG thymus. With the observations gathered during my PhD, I can develop the following scenario to potentially explain the development of MG. Initially, an unknown but stressful event induces acute thymic stress leading to massive thymocyte apoptosis. Because there are fewer thymic macrophages in predisposed MG patients, efferocytosis of apoptotic thymocytes is less efficient, and accumulating secondary necrotic thymocytes release endogenous nucleic acids. The latter activate innate signaling pathways and thymic IFN-I production. IFN- β , the orchestrator of thymic changes, thus leads to MG development. We suspect that an altered resolution of the inflammation explains the chronic IFN-I and thymic inflammation observed even long after the disease onset.