Targeting the IL-23/Th17 pathway to treat Myasthenia Gravis

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PhD thesis

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Index

List of Figures and Tables ii
Abbreviations iii
I. STATE OF THE ART 1
1. T helper 17 cells (Th17) 2
1.1. T-cell differentiation 2
1.2. Th17 cell differentiation 4
1.3. Characteristics of Th17 cells 6
1.4. Th17 cells functions in healthy individuals 9
1.5. Pathogenic and nonpathogenic Th17 cells 10
1.6. Th17 cells related cytokines 12
1.7. Th17 cells and autoimmunity 19
1.8. Therapy targeting of Th17 cells 23
2. Myasthenia gravis (MG) 28
2.1. Historical aspects of myasthenia gravis 28
2.2. Epidemiology 29
2.3. Classification of autoimmune myasthenia gravis 30
2.4. Etiology 33
2.5. Physiology of the tissues implicated in AChR$^+$ MG 36
2.6. AChR$^+$ myasthenia gravis animal models 50
3. MG therapies 52
3.1. Decision tree for treatment of MG 52
3.2. Clinical trials ongoing 56
4. Thesis aims 61
II. RESULTS 63
Article #1 64
Article #2 77
Article #3 93
III. DISCUSSION AND CONCLUSIONS 136
IV. PERPECTIVES 148
V. BIBLIOGRAPHY 150
VI. ANNEXES 179
Review # 1 180
Review # 2 189
Article contribution 198

List of Figures and Tables

Figure 1: Differentiation of T helper cells 3
Figure 2: Development of Th17 cell and its molecular mechanisms 5
Figure 3 : Classic Th17 cell 7
Figure 4 : IL-23 and IL-12 and their receptors 14
Figure 5 : IL-17 and IL-17 receptor signaling 16
Figure 6 : Autoreactive cells arise from the convergence of different factors. 20
Figure 7 : Structures of monoclonal antibodies and their potential immunogenicity 24
Figure 8 : Chemical compounds that target Th17 cells 26
Figure 9 : Potential therapeutic targets in Th17 cells 27
Figure 10 : Positive and negative selection of T-cells in the thymus 38
Figure 11 : Hyperplastic MG thymus with infiltration of B-cells 42
Figure 12 : Organization and cellular components of germinal centers 43
Figure 13 : Neuromuscular junction in physiological conditions 46
Figure 14 : Regeneration of muscle fibers 48
Figure 15 : Effects of anti-AChR antibodies in the neuromuscular junction 49
Figure 16 : Algorithm for the treatment of MG 52
Figure 17 : Mechanism of action of anti-FcRn 59
Figure 18: Schematic representation of IL-23/IL-17 loop of regulation in AChR$^+$ MG thymuses.... 140
Figure 19: Th17 cells in the development of EAMG. 141
Figure 20: Development of MG in NSG mice engrafted with AChR$^+$ MG thymus. 143

Table 1 : Immunosuppressors used in treatment of MG 54
Table 2 : Current clinical trials for the treatment of myasthenia gravis 58

Abbreviations

ACh	Acetylcholine
AChR	Acetyl choline receptor
AHR	Aryl hydrocarbon receptor
AID	Activation-induced cytidine deaminase
AIDs	Autoimmune diseases
AIRE	Autoimmune regulator
APC	Antigen presenting cells
BAFF	B cell activator factor
BATF	Basic leucine zipper transcription factor ATF-like
BCR	B-cell receptor
Blimp1	B Lymphocyte–Induced maturation protein-1
CCL	Chemokine C-C motif ligand
CCR	C-C motif chemokine receptor 6
CD	Crohns disease
CFA	Complete Freund's adjuvant
CIA	Collagen induced arthritis
CNS	Central nervous system
cTEC	cortical thymic epithelial cells
CXCL	Chemokine C-X-C motif ligand
DC	Dendritic cells
DN	Double negative
DZ	Dark zone
EAE	Experimental autoimmune encephalomyelitis
EAMG	Experimental autoimmune myasthenia gravis
eGC	Ectopic germinal center
EOMG	Early onset myasthenia gravis
ER	Estrogen receptor
ETP	Early thymic progenitors
FcRn	Neonatal Fc Receptor
FDA	Food and drug administration
FDC	Follicular dendritic cell
FoxP3	Forhead box P3
GC	Germinal center
G-CSF	Granulocyte colony stimulator factor
GM-CSF	Granulocyte-macrophages colony-stimulating factor
GPR65	G protein-coupled receptor 65
GZMB	Granzyme B
HEV	High endothelial venules
HIF1	Hypoxia induced factor 1
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL	Interleukin
IRF	Interferon-regulatory factor
IVIg	Intravenous immunoglobulins
JAK	Janus kinase
LEC	Lymphatic endothelial cells
LOMG	Late onset myasthenia gravis
LRP4	Low-density lipoprotein receptor related protein 4
LTi	Limphoid tissue inducer cell
LZ	Light zone
mAChR	Mouse acetyl choline receptor

MG	Myasthenia gravis
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cells
mTOR complex	Mammalian target of rapamycin
MuSK	Muscle-specific kinase
MyoD	Myogenic Differentiation 1
MyoG	Myogenin
NF-KB	Nuclear factor kappa B
NMJ	Neuromuscular junction
NOD	Nucleotide binding oligomerization domain containing
NRF2	Nuclear factor erythroid-derived 2-like 2
NSG	NOD SCID gamma c KO
PASI	Psoriasis area and severity index
Pax7	Paired box transcription factor 7
PBMC	Peripheral blood mononuclear cells
Pdpn	Podoplanin
PGE2	Prostaglandin E2
Poly(I:C)	Polyinosinic–polycytidylic acid
RA	Rheumatoid arthritis
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
Rgs	Regulators of G-protein signaling
RIP	Receptor-interacting protein
RORγ	Retinoic acid related orphan receptor gamma
RTE	Recent thymic emigrant
SAA	Serum amyloid A protein
SC	Satellite cells
SCID	Severe combine immunodeficient
SFB	Segmented filamentous bacteria
SGK1	Serum glucorticoid kinase 1
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
SP	Single positive
ST6gal1	ST6 -galactoside alpha-2,6-sialytransferase 1
STAT	Signal transducer and activator of transcription
TAChR	Torpedo acetylcholine receptor
Tbx21	T-box transcription factor 21
TEC	Thymic epithelial cells
Tfh	T follicular helper cell
TGF-β	Transforming growth factor beta
Th	T helper cell
TLO	Tertiary lymphoid organ
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TREC	T cell receptor excision circle
Treg	Regulatory T cells
TSA	Tissue specific antigen
TYK	Tyrosine kinase
VDR	Vitamin D receptor

I. STATE OF THE ART

1. T helper 17 cells (Th17)

In the early 2000s, studies advanced the well-established paradigm of T helper 1/T helper 2 (Th1/Th2) by identifying another subpopulation of $CD4^+$ effector T-cells that express Interleukin 17 (IL-17) and that require interleukin 23 (IL-23p19) and not interleukin 12 (IL-12p40) for their development [1-4]. These characteristics were not consistent with the well-known Th1 cells that express IFN-γ and require IL-12 to differentiate or the Th2 cells that express IL-4 [5]. Thus, these observations led to discovery of an undefined subpopulation of $CD4^+$ T helper cells known as Th17 due to their capacity to express IL-17 as their main cytokine.

Since the discovery of Th17 cells, other subpopulations of effector T-cells have also been described. These subpopulations present different characteristics in their transcription factors, the cytokines required for their differentiation as well as their cytokine production

1.1. T-cell differentiation

The immune system is a highly ordered system that relies on the balance between pro-inflammatory and anti-inflammatory signals and cellular responses. $CD4^+$ T-cells are essential in the adaptive immune system. The different $CD4^+$ T helper cell subpopulations arise from the activation of naïve T-cells by antigen-presenting cells (APC) and the presence of specific cytokines in the microenvironment. T-cell activation by APC is carried on by the interaction between T-cell receptor (TCR) and peptides presented by APC in the context of major histocompatibility complex (MHC) class-II [6]. The initial activation of $CD4^+$ T-cells is followed by co-stimulatory signals that come from the interaction of CD28 on T-cells and CD80 or CD86 on APC. This signaling promotes a great proliferation of T-cells that, under the controls of cytokines, differentiate into specific subpopulations of effector T-cells.

$CD4^+$ effector T-cells are categorized according to the cytokines required for their differentiation, their expression of specific transcription factors and their secretion of cytokines. To date, the identified subpopulations of T-cells are Th1, Th2, Th17, Th9, Th22, Tfh and Treg cells **(Figure 1)**.

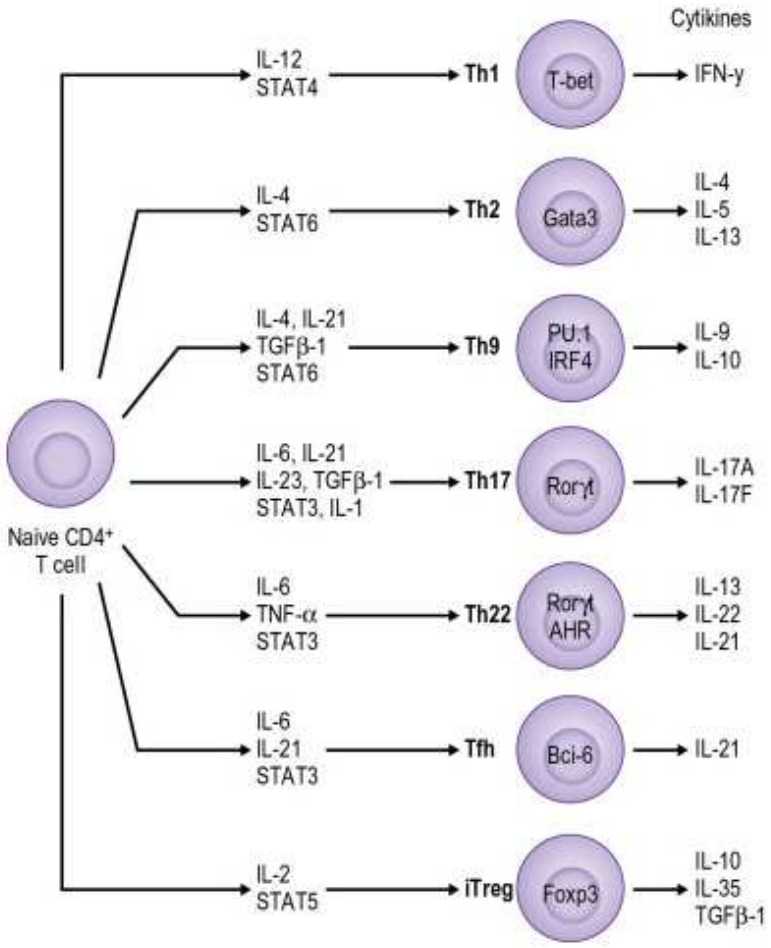

Figure 1: Differentiation of T helper cells

Naïve T-cells can differentiate into different subpopulations according to the cytokines present in their environment. The subpopulations of T-cells are characterized by their expression of transcription factors and their production of specific cytokines [7].

Th1 cells are characterized by the expression of IFN-γ and require the presence of IL-12 to differentiate. Their master transcription factor is T-bet. Th1 cells are linked to clearance of intracellular pathogens [8]. Th2 cells are normally produced in response to extracellular pathogens such as bacteria and helminths. Th2 cells are characterized by the production of IL-4, which is also the cytokine required for their initial differentiation. Th2 cells' main transcription factor is GATA3 [9].

Th9 and Th22 are two subpopulations of effector T-cells defined by their respective expression of IL-9 and IL-22 [10, 11]. Th9 transcription factor is PU.1. Differentiation of Th9 cells requires the

presence of IL-4 and TGF-β1 [11]. Th22 cells present aryl hydrocarbon receptor (AHR) as their main transcription factor, and their differentiation relies on the presence of IL-6 and TNF-α [10]. Th22 cells' function is to protect from infection in barrier tissues [12].

Follicular T helper cells (Tfh) are a subpopulation of T-cells that produce IL-21 as their main cytokine. Tfh cells are required for the activation of B-cells within germinal centers. Tfh transcription factor is Bcl-6. Development of these cells requires the presence of IL-6 and IL-21 [13].

Regulatory T-cells (Treg cells) are critical $CD4^+$ T-cells, as they have the ability to control effector T-cells. The Treg cell transcription factor is FoxP3. Development of Treg cells requires the presence of IL-2 and TGF-β1 [14]. Treg cells are characterized by their expression of anti-inflammatory cytokines, including IL-10 and TGF-β1, and their main function is maintenance of immune tolerance [15].

As describe in detail in the following sections, Th17 cells require the presence of IL-6, TGF-β1, IL-1β and IL-23 to differentiate. Th17 cells are characterized by their expression of RORγt as their master transcription factor, and they can produce cytokines such as IL-17, IL-21 and IL-22 along with granulocyte macrophage colony-stimulating factor (GM-CSF). Th17 cells are required for clearance of pathogens in barriers tissues. However, their implication as the main inducers of autoimmune diseases has revealed the complexity of their physiology.

1.2. Th17 cell differentiation

The process of differentiation of naïve $CD4^+$ T-cells into Th17 cells is now well understood. It is orchestrated in consecutive overlapping steps, differentiation, amplification and stabilization that result in nonpathogenic or pathogenic Th17 cells **(Figure 2)** [16, 17].

1.2.1. Differentiation and amplification

Naïve T-cells in the presence of transforming growth factor beta 1 (TGF-β1) and IL-6 differentiate into the Th17 cell phenotype [18, 19] through the Janus-kinase/signal transducer and activator of transcription 3 (JAK/STAT3) transduction pathway initiated at the IL-6 receptor (IL-6R). STAT3 induces the expression of RORγt and downregulates TGF-β-induced FoxP3 [18, 20]. RORγt controls the expression of the signature cytokines and membrane receptors of Th17 cells (i.e., IL-17A, IL-17F,

IL-22, GM-CSF, CCR6, CCR4, CCL20 and IL-23R) [21, 22]. Then IL-21, IL-1β and endogenously produced TGF-β3 amplify the Th17 cell phenotype, which is then stabilized by IL-23 [16, 22].

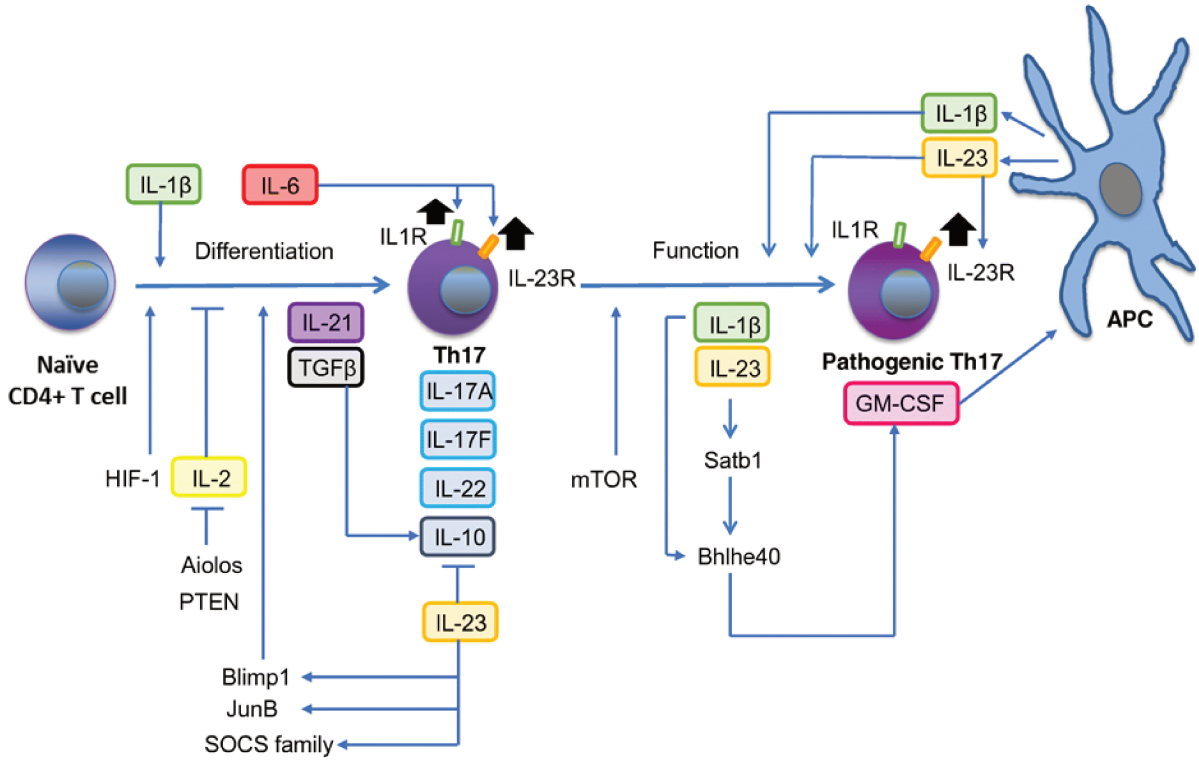

Figure 2**: **Development of Th17 cell and its molecular mechanisms

The molecular mechanisms of Th17 cell differentiation are controlled by the presence of IL-6, IL-1β, TGFβ1/3 and IL-23. Under the control of these cytokines and other environmental factors, Th17 cells express transcription factors such as STAT3 and RORγ. The phenotype is sustained by the co-expression of HIF1, mTOR, Blimp1 and JunB, transcription factors that enhance the expression of RORγ and IL-23R as well as the production of IL-17, IL-22, IL-21 and GM-CSF[17].

The molecular mechanisms in the differentiation process of Th17 cells include different molecules that interact to reinforce the Th17 phenotype. For instance, activation of STAT3 by IL-6 and TGF-β1 induces the expression of hypoxia-inducible factor 1 (HIF-1α). HIF-1α is able to bind to the promoter region of RORγt and enhances its expression [23] while reducing protein levels of FoxP3 [23, 24]. Interferon regulatory factor (IRF4) and Basic leucine zipper transcription factor ATF-like (BATF) are two essential transcription factors in the initial development of Th17 cells [25, 26]. They contribute to the initial chromatin accessibility after TCR activation and to the recruitment of RORγt [27].

1.2.2. Stabilization

IL-23 signaling, through IL-23R, is required to stabilize Th17 cells. Signaling through IL-23R, IL-23 reactivates STAT3 and induces the expression of B lymphocyte–induced maturation protein-1 (Blimp1) [28]. Blimp1 directly binds to IL-23R and IL-17 loci and colocalizes with RORγt and STAT3, which enhances the expression of IL-17 and GM-CSF [28].

IL-23 is a cytokine composed of IL-12p40 and IL-23p19 subunits. Of note, IL-12p40 heterodimerizes with IL-12p35 to induce the differentiation of Th1 cells, while IL-23p19 specifically controls the development of Th17 cells. IL-23 is not required for the initial development of Th17 cells, but it is critical for their complete differentiation [22]. Therefore, IL-23 is seen as a main inducer of pathogenicity and is essential in the pathological process of autoimmunity.

JunB, an AP-1 transcription factor is potentially overexpressed in Th17 cells [29]. A deficiency of JunB in developing Th17 cells impairs the binding of STAT3 to RORc and IL-17a loci [29]. JunB facilitate the maintenance of DNA binding of BATF, IRF4 and STAT3 onto Th17 genes including RORγt [29]. JunB is not required for the initial development of Th17 cells, since its effects are IL-23-dependent [30]. JunB also downregulates FoxP3, Tbx21 and IFN-γ. Therefore, JunB controls the differentiation of Treg cells or Th1 cells [30, 31] and reinforces the Th17 cell phenotype. Similarly, the transcriptional regulator RBPJ, a canonical Notch signaling molecule, is expressed during the differentiation process of Th17 cells by reinforcing IL-23R expression and repressing IL-10 expression [32].

The mammalian target of rapamycin complex 1 (mTOR1) has also been involved in Th17 cell development. The PI3K/mTOR1 axis controls the differentiation of Th17 cell by adjusting the translocation of RORγ into the nucleus [33].

1.3. Characteristics of Th17 cells

Th17 cells derive from $CD4^+$ nondifferentiated T-cells into $CD4^+$ activated and specialized T-cells that display specific characteristics. Th17 cells express the signal transducer and activator of transcription 3 (STAT3) and retinoic acid related orphan receptor gamma (RORγt) as their master transcription factors [34]. At the membrane, Th17 cells are characterized by the expression of the chemokine receptors 6 and 4 (CCR6, CCR4) and the interleukin receptor 23 (IL-23R). Finally, the classic cytokines produced by the Th17 cell are IL-17A/F and IL-21 [34-36]. However, Th17 cells can produce other

cytokines, such as GM-CSF [37], IL-22 [38] and IFN-γ [35]. Therefore, Th17 cells can be defined as $CD4^+STAT3^+ROR\gamma^+CCR6^+CCR4^+IL23R^+IL\text{-}17^+$ cells **(Figure 3)**.

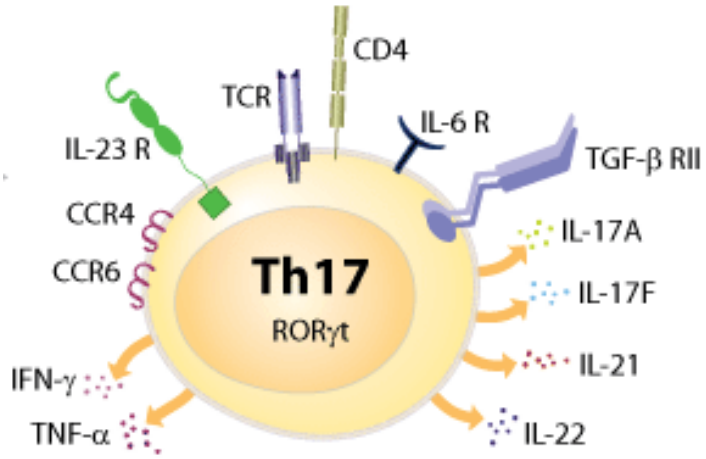

Figure 3** : **Classic Th17 cell
Th17 cells are identified by their expression of transcription factor (RORγ), chemokines and cytokines receptors (CCR6, CCR4, IL23R) and the production of cytokines (IL-17A/F, IL-21, IL-22) [39].

Plasticity and transdifferentiation of Th17 cells

One enigmatic T-cell feature is plasticity. This phenomenon refer to their ability to acquire the functional capabilities of another population while conserving their fundamental program. Accumulating data have shown that Th17 cells are chameleon cells that can express genes classically "restricted" to other T-cell lineages with whom they share initial development steps. These genes include STAT4 for Th1, CCR4 for Th2, AhR for Th22, PU.1 for Th9 and FoxP3 for Treg cells [11, 40-42].

Studies have shown that Th17 cells expressing RORγt can acquire Th1 capabilities and express IFN-γ, the classical Th1 cytokine [43]. This change in phenotype can be dictated by the microenvironment. For instance, low concentration of IL-23 or increase of IL-12 and TNF-α in an inflammatory milieu limits Th17 cell expansion and directs the emergence of IFN-γ producing cells. Indeed, these cells, called Th1-like ex-Th17 cells, are considered to be cells that promote autoimmunity [43].

Th17 cells can also acquire a Treg phenotype. Plastic changes from Th17 cells to more anti-inflammatory cells (Treg-like) depend on AHR and TGF-β signaling [40]. An example of the capacity of T-cells to change their "classic phenotype" is observed in patients affected by inflammatory bowel disease (IBD). IBD patients present an increased number of $FoxP3^+$ cells expressing $IL\text{-}17^+$ ($FoxP3^+IL17^+$) [44]. Inversely, it has also been shown that $FoxP3^+$ Treg cells can express IL-17 in the presence of IL-1β and IL-6. However, $Foxp3^+$ Treg cells in the presence of IL-1β, IL-6 and TGF-β reduce expression of IL-17 [45]. Interestingly, Treg cells that express IL-17 can conserve their suppressive capacity when the inflammatory conditions are reversed [45]. This shows that the plastic characteristics are not limited to Th17 cells and that Treg cells can participate in the inflammatory processes when stimulated by the microenvironment.

T follicular helper cells (Tfh) are a set of effector T-cells that participate in B-cell differentiation within the germinal centers. Th17 cells have also the ability to transdifferentiate into Tfh. Hirota et al. showed that in Peyer's patches, Th17 cells can change their phenotype and upregulate Bcl6 and IL-21, which are classic markers of Tfh cells. Of note, transdifferentiation of Th17 cells to Tfh cells is independent of IL-23 [46].

Th22 and Th9 are two recently described T-cell subpopulations that are closely related to Th17 cells. IL-22 is a cytokine that acts in mucosal surfaces and was originally thought to be secreted concomitantly with IL-17A by Th17 cells [47]. It is now known that Th22 cells produce IL-22 independently of IL-17 [47]. The differentiation processes of Th17 and Th22 cells share IL-6 as a key cytokine in their development [10, 12]. However, TGF-β, a cytokine required for Th17 cell differentiation, inhibits Th22 cell differentiation [12]. Th17 and Th22 also share AHR as transcription factor. AHR and T-bet are considered as the classical transcription factors of Th22 cells [48]. In Th17 cells, AHR is overexpressed in the nonpathogenic Th17 cell phenotype [22]. Moreover, stimulation of differentiated Th17 cells with AHR agonists induces the expression of IL-22 [48]. Therefore, trans-differentiation between Th22 and Th17 might be driven by the concentration of TGF-β in an inflammatory microenvironment, while the production of IL-22 is linked to AHR activation. Th22 cells have been also implicated in enteric protection from infection and in skin immune balance [12, 47].

Th9 cells are characterized by their production of IL-9, and their differentiation relies on the presence of IL-4 and TGF-β1 [11]. Before the discovery of Th9 cells, IL-9 production was attributed to Th2 cells [49]. Today, there are studies showing that, in addition to being produced by Th9, IL-9

can be produced by Th2 cells, Th17 cells [50], mast cells [51] and natural Treg cells (nTreg) [52]. Interestingly IL-9 can promote survival and increase the suppressive capacity of Treg cells [53]. Similarly, to Th17 cells, Th9 cells require TGF-β to differentiate, which can induce the expression of FoxP3. This expression is controlled by IL-4 signaling that activates STAT6 and blocks FoxP3 expression [54]. Of note, the production of IL-9 by Th17 cells is reduced when stimulated with IL-23, and a lack of IL-23R increases production of IL-9 [53]. Like Th17 cells, Th9 cells have also been linked to the development of psoriasis, IBD and multiple sclerosis (MS) [55, 56]

1.4. Th17 cells functions in healthy individuals

The first and main identified physiological function of Th17 cells is to protect mucocutaneous barriers from infection. IL-17 producing cells are normally found in mucosal barriers such as the gut, the skin and the lungs. The relevance and necessity of Th17 cell presence in these zones have been demonstrated with patients affected by primary immunodeficiencies related to Th17 cells. For instance, individuals with autosomal gene deficiency in IL-17F and IL-17RA [57], with a gain-of-function mutation in the STAT1 gene [58], or with a mutation on the STAT3 gene [59] display decreased or null activation of Th17 cells, which engenders chronic mucocutaneous candidiasis or recurrent infections of *Staphylococcus aureus*. These observations reveal that Th17 cells and the IL-17 signaling pathway are essential in the protection against infections under physiological conditions.

Numerous studies have shown that under physiological conditions, Th17 cells arise in the gut, induced by segmented filamentous bacteria (SFB) [60-63]. In mice, colonization of the ileum by SFB stimulates expression of serum amyloid A protein 1 and 2 (SAA1/2) by epithelial cells [64]; in parallel, dendritic cells present SFB-related antigens to $CD4^+$ T-cells to prime them into a Th17 cell phenotype [62]. Meanwhile, expression of SAA1/2 activates monocyte-derived cells to produce IL-23 [64]. The global microenvironment promotes, then, the active differentiation of protective Th17 cells within the lamina propria of the small intestine [65]. Of note, SFB induced Th17 cells in homeostatic conditions acquire a regulatory phenotype with high expression of IL-10 in the resolution of the inflammation [40].

Reciprocally, production of IL-17 by Th17 cells controls the expansion of commensal bacteria. IL-17 signals through IL-17R in epithelial cells to induce the production of anti-microbial peptides such as

regenerating-islet derived Reg(3) and α-defensins[65]. Therefore, under physiological conditions, there is a reciprocal symbiotic regulation between commensal bacteria and protective Th17 cells.

Similar to the phenomenon observed in the gut, protection against infections in the lung relies on the induction of Th17 cells by bacteria. For instance, nasopharyngeal colonization in early life by *Streptococcus pneumoniae* induces the differentiation of Th17 cells and the overexpression of IL-17 and IL-22. Both cytokines are able to stimulate epithelial cells to recruit innate immune cells and promote bacterial clearance [66-68]. Interestingly, nasopharyngeal colonization by *S. pneumoniae* is thought to be protective against further infections in the lung and relies on Th17 cells [67]. Moreover, Th17 cells are also involved in the lung defense against other microorganisms, including *Staphylococcus aureus* [69], *Bordetella pertussis* [70] and *Mycobacterium tuberculosis [71].*

Thus, Th17 cells arise in the mucosal barriers due to stimuli provided by commensal bacterial. In these tissues, Th17 cells are essential for host protection. Nevertheless, Th17 cells and commensal bacteria coexist in a tight equilibrium that, if broken, might promote chronic inflammation and lead to autoimmune diseases.

1.5. Pathogenic and nonpathogenic Th17 cells

Today it is accepted that Th17 cells can be differentiated into pathogenic and nonpathogenic Th17 cells [22, 72]. Nonpathogenic Th17 cells express signature genes such as IL-10, IL-9 and AHR [22]. In contrast, pathogenic Th17 cells present an overexpression of genes that include CXCL13, IL-22, GZMB, IL-7R, TBX21 (T-bet) and STAT4. Hence, pathogenic and nonpathogenic Th17 cells can be considered as two different subpopulations with specific molecular signatures.

This diversity in maturation is established according to the cytokine milieu that sustains the differentiation process. For instance, Th17 cells differentiated with IL-6 and TGF-β1 and in the absence of IL-23 produce IL-10, in addition to IL-17 [73]. In this context, Th17 cells are consider as nonpathogenic. Studies in vivo have shown that in a limited inflammatory context, Th17 cells acquire a regulatory phenotype and participate in the resolution of the inflammation [74]. This acquired characteristic is achieved under the influence of TGF-β1 and the consequent activation of AHR [40].

In contrast, development of pathogenic Th17 cells requires IL-23 in addition to IL-6 and TGF-β1. Langrish et al. showed that IL-23p19 deficient mice do not develop experimental autoimmune encephalomyelitis (EAE) illustrating the IL-23p19 requirement for the pathology's development [1].

Another study showed that IL-23R$^{-/-}$ T-cells are still able to produce IL-17, although at a lower level compared to wild-type T-cells, and IL-23R$^{-/-}$ T-cells did not promote clinical signs of EAE [75]. These results strongly suggest that IL-23 controls the development of Th17 cells to acquire the pathogenic phenotype that sustains autoimmune diseases. Moreover, TGF-β3 has also been implicated in the induction of pathogenic Th17 cells [22]. In fact, Th17 cells that develop with IL-23 produce TGFβ-3 that, in combination with IL-6, induces a characteristic transcriptional signature of pathogenic Th17 cells [22].

Pathogenic Th17 cells are inducers and sustainers of autoimmune diseases. Therefore, studies have focused on potential pathogenic markers that could govern the development of these cells and determine the signaling pathway. For instance, serum glucocorticoid kinase 1 (SGK1), a kinase usually known as a sensor of NaCl homeostasis, has been linked to Th17 cells. Indeed, the IL-23-induced differentiation of Th17 cells in a high-salt milieu upregulates the expression of SGK1 [76]. SGK1 regulates the expression of IL-23R through the inactivation of Foxo1, a transcription factor that inhibits IL-23R [77]. Another molecular controller of Th17 cells is CD5L. CD5L, usually expressed in macrophages, is a protein involved in lipid metabolism that can be overexpressed under inflammatory conditions [78]. In Th17 cells, CD5L was found to be expressed predominantly by nonpathogenic Th17 cells. CD5L is expressed in the initial step of the differentiation of Th17 cells under the control of STAT3 [79]. There, CD5L controls the content of fatty acids and the production of endogenous ligands of RORγ. This results in decreased RORγ binding to IL-17 and IL-23R regions and enhanced binding to anti-inflammatory IL-10 [79]. Hence, expression of CD5L can be considered as a mechanism of self-regulation of Th17 cells through the metabolism of lipids [79].

G protein-coupled receptor 65 (GPR65) is a membrane receptor that senses extracellular protons. It is expressed in lymphocytes and lymphoid tissues [80]. GPR65 single nucleotide polymorphisms (SNP) have been associated with development of MS, IBD and Chron's disease [81-83]. In vitro analyses have shown that lack of GPR65 on T-cells reduces the production of IL-17 by Th17 cells [84]. As with GPR65, a deficiency of TOSO, a Fas-mediated apoptosis inhibitory molecule [85], significantly reduces the production of IL-17 in Th17 cells [84]. Hence, in contrast to CD5L, GPR65 and TOSO are part of the pro-inflammatory program of Th17 cells and induce a pathogenic phenotype.

As previously mentioned, the main function of Th17 cells is clearance of external pathogens. Nucleotide-binding oligomerization domain-containing 1 and 2 (NOD1 and NOD2) are bacterial intracellular sensors that induce inflammation through NF-κB after the recruitment of receptor-

interacting protein 2 (RIP2) [86]. Loss of RIP2 has been involved in reduced bacterial clearance and chronic inflammation. In Th17 cells, loss of RIP2 promotes a pathogenic phenotype of Th17 cells through RORα signaling and not RORγt [87]. Therefore, RIP2 might act as a regulator of pathogenic Th17 cells.

1.6. Th17 cells related cytokines

1.6.1. Cytokines involved in Th17 cell differentiation

Development of Th17 cells relies on the presence of specific cytokines throughout the differentiation, amplification and stabilization processes. Thus, each cytokine entails phenotypical changes leading naïve T-cells to acquire a Th17 cell phenotype.

1.6.1.1. Interleukin 6

IL-6 is a multifunctional cytokine involved in organ development, inflammation and immune responses [88]. The pleiotropic functions of IL-6 are mediated through its membrane receptor (mIL6R), the classical signaling route, or through its soluble receptor (sIL-6R). Both signaling pathways require interaction with the glycoprotein 130 (gp130). IL-6R is expressed in T-cells, monocytes and activated B-cells. IL-6 is crucial in the development of Th17 cells, as it enhances the initial expression of STAT3, which promotes RORγt expression [89]. IL-6 is an essential cytokine to mediate the balance between Treg and Th17 cells. IL-6 signaling represses TGF-β-induced Foxp3 expression during Th17 cell differentiation [90]. In autoimmune diseases such as MS, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and myasthenia gravis (MG), IL-6 is overproduced [91-93]. IL-6-deficient mice have been shown to be protected against EAE, collagen-induced arthritis (CIA) and experimental autoimmune myasthenia gravis (EAMG), inducible mouse models of MS, RA and MG respectively, suggesting an involvement of IL-6 in disease development [94-96].

1.6.1.2. Transforming growth factor beta (TGF-β)

The transforming growth factor (TGF) is a superfamily of cytokines that includes three isoforms: TGF-β1, TGF-β2 and TGF-β3 [97]. The TGF-β is a secreted protein that regulates proliferation and differentiation of different cell types. TGF-β can be produced by all immune cells, including T-cells and B-cells, and suppresses the growth and the differentiation of these cells [98]. TGF-β signals via

two serine/threonine kinase receptors (TβRI and TβRII) to activate the Smad transcription factors family, specifically SMAD2 and SMAD3 [99]. TGF-β displays a wide spectrum of effects and participates in the balance between pro-inflammatory and anti-inflammatory microenvironments [100]. For instance, induced Treg (iTreg) requires the presence of TGF-β1 to express FoxP3 and CD25 [101], while TGF-β1 in combination with IL-6 promotes Th17 cell differentiation [19].

TGF-β1 may be produced in an autocrine manner throughout the differentiation of Th17 cells [102]. However, pathogenicity of Th17 cells is induced by TGF-β3 [22]. Like TGF-β1, TGF-β3 signals through TβRI and TβRII, but it preferentially activates SMAD1 and SMAD5, whereas TGF-β1 activates SMAD2 and SMAD3 [103]. Of note, experiments using TGF-$\beta^{-/-}$ mice illustrate the indispensable role of this cytokine. Lack of TGF-β induces, in animals, severe inflammation and multiorgan autoimmunity and greatly reduces lifespan [104, 105].

Hence, TGF-β1 is an indispensable cytokine that control the balance between pro-inflammatory and anti-inflammatory signals (i.e., between Th17 and Treg cells) in the immune system, while TGF-β3 is a potentiator of inflammation by controlling the pathogenicity of Th17 cells.

1.6.1.3. Interleukin 1 beta (IL-1β)

IL-1β belongs to the IL-1 family that includes IL-1α, IL-1Ra and IL-1β [106]. IL-1β signals through interleukin-1 receptor 1 (IL-1R1). This receptor is promoted by IL-6 in developing T-cells. Signaling through IL-1R1 induces the expression of IRF4 and RORγt and induce the expression of IL-17 [107]. Moreover, IL-1β signaling phosphorylates mTOR in Th17 cells and therefore participates in the proliferation of these cells [108]. IL-1β has also been shown to be able to alter FoxP3 and induce a switch of Treg into a Th17 phenotype [109].

1.6.1.4. Interleukin 23 (IL-23)

IL-23 is a heterodimeric cytokine composed of two subunits (IL-23p19 and IL-12p40). IL-23 was first discovered in the early 2000s with computational analysis that highlighted its close resemblance to IL-12p40, a subunit shared with IL-12p35 (IL-12) and related to Th1 cell development [110].

The main described producers of IL-23 are macrophages and dendritic cells in response to toll-like receptor (TLR) activation by exogenous and endogenous signals [111-113]. Furthermore, activation of TLR4 also induces IL-23 production by epithelial cells such as keratinocytes [114].

IL-23, in its dimeric form IL-12p40/IL-23p19 (IL-23), signals through the IL-23 receptor and the IL-12 receptor beta 1 (IL-23R and IL-12Rβ1) [115]. These receptors are expressed on dendritic cells, macrophages, natural killer cells (NK cells) and activated T-cells [116]. In dendritic cells, IL-23 stimulation promotes antigen presentation [117].

Activation of IL-12R signaling induces different functions compared to activation of IL-23R. Signaling through the IL-12 receptor induces the phosphorylation and activation of STAT4, leading to the differentiation of Th1 cells and the expression of IFN-γ [115]. In contrast, activation of IL23R signaling triggers STAT4 and the phosphorylation and activation of STAT3, promoting the expression of RORγt and the consequent expression of IL-17 [115, 118] **(Figure 4)**.

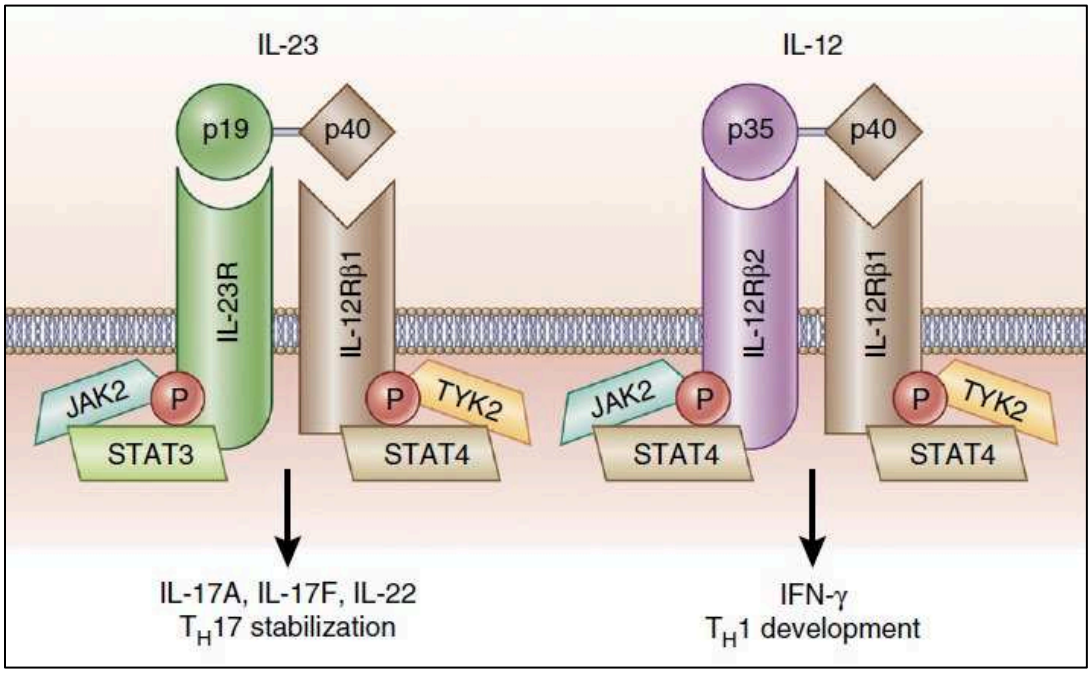

Figure 4 : IL-23 and IL-12 and their receptors

IL-23 and IL-12 are related cytokines with different effects. These cytokines have a common subunit, IL-12p40, that dimerizes with IL-12p35 or IL-23p19 to conform IL-12 or IL-23, respectively. IL-12 signals through IL-12Rβ1 and IL-12Rβ2, while IL-23 signals through IL-12Rβ1 and IL-23R. Activation of IL-12R induces the phosphorylation of STAT4 and production of IFN-γ, while IL-23R phosphorylates STAT4 and STAT3 to induce the production of IFN-γ and IL-17 [118].

Of note, IL-23R expression by Th17 cells depends on IL-6, IL-1β and TGF- β. These cytokines induce the expression of RORγt, which promotes IL-23R expression [34, 119]. Therefore, IL-23 acts on Th17 cells as a retro-positive loop that stabilizes differentiation of Th17 cells and as an enhancer of RORγt, IL-17, IL-21, IL-22 and GM-CSF expression [120].

The critical role of IL-23 has been demonstrated in mouse models of RA and MS. Indeed, deficiency of IL-23, although not of IL-12, protects mice from development of CIA and EAE [4, 36]. In humans, pathologies such as RA, MS, SLE and psoriasis present an increased production of IL-23 [121-123]. Emerging therapies that target IL-23 are now available for treatments of psoriasis and Crohn's disease, corroborating the significant role of this cytokine in the development of human autoimmune diseases [124, 125].

1.6.2. Cytokines produced by Th17 cells

1.6.2.1. Interleukin 17 (IL-17)

IL-17 belongs to a family of cytokines that comprises six members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F). IL17A and IL17F are the two cytokines best understood and investigated. IL-17A was described in 1996 and was first known as cytotoxic T-lymphocyte-associated antigen 8 (CTLA8) [126]. Fossiez et al. showed that activated $CD4^+$ cells are the main producers of IL-17 in peripheral blood mononuclear cells (PBMC). Moreover, secreted IL-17 is able to induce the production of IL-6, IL-8, prostaglandin E2 (PGE2) and granulocyte colony-stimulating factor (G-CSF) by synovial fibroblasts, skin fibroblasts, brain endothelial cells, embryonic lung fibroblasts, kidney carcinoma and epithelial cells, illustrating the important inflammatory role of IL-17 in autoimmune diseases [126, 127].

Of note, IL-17A and IL-17F are the main cytokines produced by Th17 cells. IL-17A and IL-17F share 50% homology in their sequence [128]. They can act as homodimer and/or heterodimer [128]. Expression of both cytokines is under the control of the nuclear receptor RORγt [34]. However, IL-17A displays the higher inflammatory capacity [128], although both cytokines can synergize with TNF-α to increase the inflammatory response [129].

IL-17 signals through the IL-17 receptor (IL-17R). The IL-17R family includes five members: IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE [127, 130]. IL-17RA is ubiquitously expressed in many cells and is the common subunit that interacts with other members to form heterodimers. For instance, IL-17RA and IL-17RC form a classical heterodimer to respond to IL-17A and IL-17F [130]. IL-17RA signaling recruits the E3 ubiquitin ligases Act1 and TNF receptor-associated factor 6 (TRAF6) that activate the NF-κB pathway [131, 132] to induce the expression of inflammatory genes such as G-CSF and chemokines including CXCL1, CXCL2, CXCL8 and notably CCL20, the chemoattractant

molecule of CCR6^{+} cells (Th17 cells) [126, 133] (**Figure 5**). Of note, a mechanism for negative regulation of IL-17 signaling has been identified through TRAF3 and TRAF4. On one side, TRAF3 inhibits the interaction between IL-17R and Act1, while TRAF4 competes with TRAF6 to interact with Act1 [134, 135] (**Figure 5**).

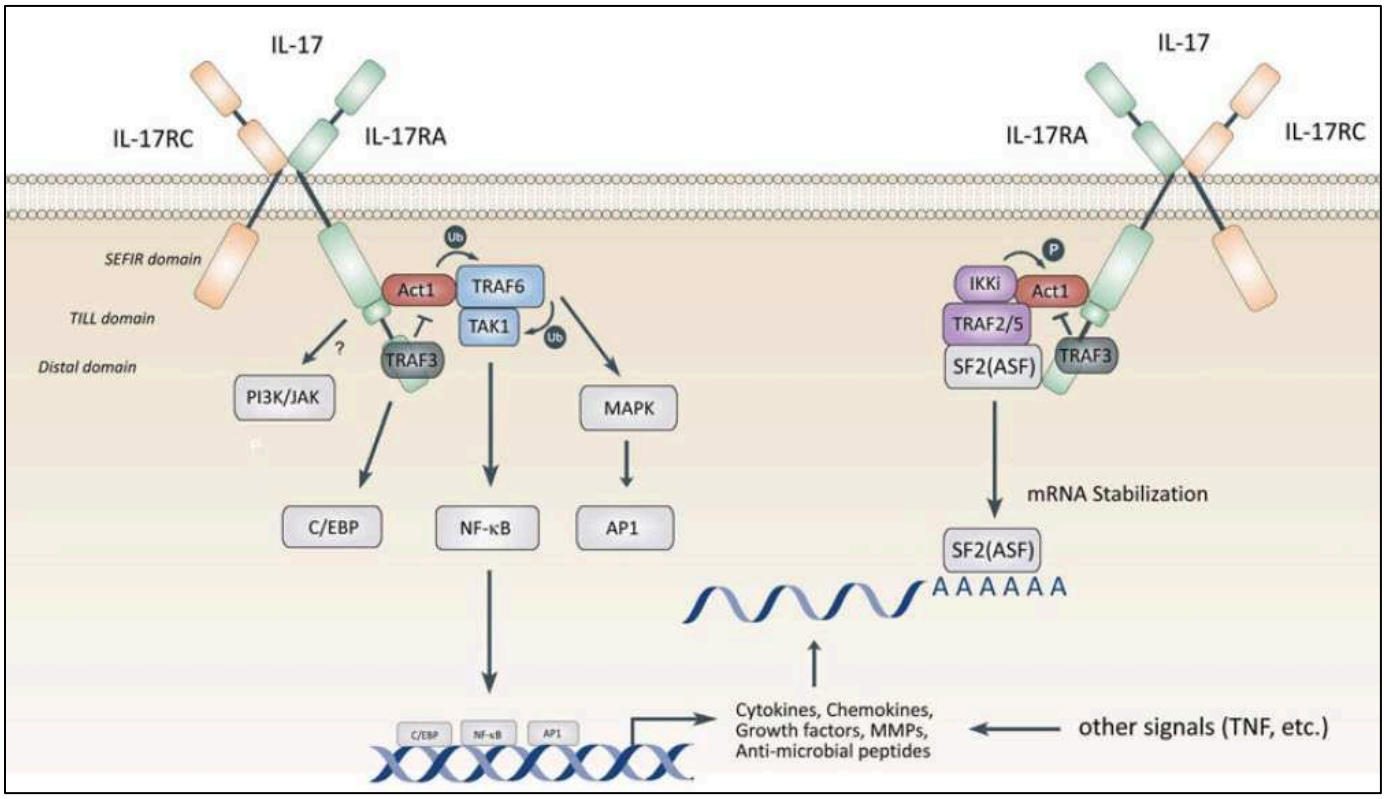

Figure 5 : IL-17 and IL-17 receptor signaling

The inflammatory effects of IL-17 are mediated by IL-17RA/C. Activation of IL-17R recruits Act1 to induce the ubiquitination of TRAF6 and TAK1 and activate NF-κB, a transcription factor that promotes the expression of pro-inflammatory cytokines (IL-6 and GM-CSF), chemokines (CXCL2, CXCL5 and CCL20) and antimicrobial peptides (β-defensins). IL-17R signaling also stabilizes mRNA through TRAF2/5. Modulation of IL-17R downstream signaling is achieved through TRAF3, which avoids Act1 recruitment and TRAF4, which directly binds to Act1 and blocks Act1-TRAF6 interaction [136].

Unlike IL-17A and IL-17F, the other members of the IL-17 family (i.e., IL-17B–D) are still not well known. IL-17B and IL-17C are expressed in the spinal cord, pancreas, small intestine, stomach and the colon and harbor specific receptors IL-17RB and IL-17RE, respectively [137, 138]. Moreover, IL-17B has been linked to autoimmune diseases, since it is overexpressed in the active phase of SLE

and in neutrophils found in the synovial tissue of RA patients [138, 139]. IL-17C is expressed by epithelial cells after TLR activation [137, 140, 141].

IL-17D is implicated in tumor rejection through the recruiting of NK cells and activation of macrophages [142]. In mice, infection with cytomegalovirus induces the production of IL-17D by fibroblasts [143]. IL-17D is also produced through activation of the transcription factor nuclear factor erythroid-derived 2-like factor 2 (NRF2), which is inducible by oxidative stress [144]. Finally, IL-17E (also known as IL-25) is a cytokine implicated in the regulation of Th2 in parasite infections and allergies [137]. IL-17E is expressed by epithelial cells, innate immune cells and Th2 T-cells [145]. It signals through IL-17RA and IL-17RB, which are expressed in endocrine tissue and Th2 cells [146]. IL-17E can also reduce the production of IL-23 by dendritic cells and therefore limits Th17 cell development [127].

1.6.2.2. Interleukin 21 (IL-21)

IL-21 is a pleiotropic cytokine produced by Tfh, NK cells and Th17 cells. It is part of a family of type I four-α-helical-bundle cytokines that includes IL-2, IL-4 and IL-7 [147, 148]. IL-21 expression is modulated by aryl hydrocarbon receptor nuclear translocator-like 2 (Arntl2), which blocks the IL-21 gene start codon to prevent RNA polymerase II binding [149]. IL-21 signals through its cognate receptor IL-21 receptor (IL-21R) [150]. IL-21R activates the JAK1/3 signaling pathway and induces the expression of STAT3, which in Th17 cells promotes the expression of IL-23R and IL-17A [119]. More, IL-21 activates IRF4 and promotes IL-17 expression by RORγt and RORα while inhibiting FoxP3 expression [151]. IRF4 can also directly promote the expression of IL-21 in Th17 cells [152]. In contrast, IL-21 may also induce the expression of suppressor of cytokine signaling 3 (SOCS3), which is a negative regulator of STAT3 [153]. Therefore, IL-21 can act as a mechanism of control in the differentiation of Th17 cells. Furthermore, Th17-produced IL-21 and IL-22 have been shown to be involved in the control of the pathogenic autoantibodies by controlling the expression of ST6-galactoside alpha-2,6-syalytranferase 1 (ST6gal1), an enzyme expressed on B-cells that controls IgG glycosylation [154]. Furthermore, IL-21 production by Th17 cells and Tfh cells acts on B-cells present in germinal centers to induce the differentiation of B-cell receptor-activated B-cells into plasma cells through the activation of STAT3 and Blimp1 [155].

1.6.2.3. Interleukin 22 (IL-22)

IL-22 belongs to the IL-10 cytokine family. It is mainly produced by Th22 cells and Th17 cells but also by innate immune cells, including NK cells and lymphoid tissue inducer cells (LTi) [156, 157]. Production of IL-22 by Th22 cells and Th17 cells is controlled by AHR [158]. IL-22 signals through its heterodimeric receptor conformed by IL-22 receptor α1 and IL-10 receptor 2 (IL-22Rα1 and IL-10R2) [159]. IL-22Rα1 is expressed in most nonhematopoietic cells, such as keratinocytes, endothelial cells and epithelial cells [89]. Signal transduction of IL-22Rα1 activates the MAPK and STAT3 pathways that upregulate genes involved in cell survival and differentiation, such as Bcl-2, cyclin D1 and c-Myc, especially in epithelial cells [160, 161]. Moreover, IL-22 induces the expression of antibacterial peptides in epithelial cells of barrier tissues, such as the lung, the skin and the intestine [162, 163]. IL-22 bioavailability is regulated by a soluble protein called IL-22 binding protein (IL-22BP) that displays a higher affinity to IL-22 than the IL-22Rα1 and therefore acts as an endogenous antagonist [164, 165]. Due to the variety of effects in non-hematopoietic cells, IL-22 is considered as a cytokine that links inflammatory T-cells with other nonimmune cells.

1.6.2.4. Granulocyte macrophage colony-stimulating factor (GM-CSF)

GM-CSF is a cytokine that stimulates the proliferation of hematopoietic cells [166, 167]. GM-CSF is mainly produced by NK cells, Th17 cells and innate lymphoid cells. GM-CSF can also be produced by lung epithelial cells, especially after they encounter allergens [168], and by synovial fibroblasts in presence of high levels of IL-1β and TNF-α [169]. GM-CSF receptor (GM-CSFR), expressed in most of the innate immune cells, is constituted of a specific GM-CSF α chain and a signal-transducing β subunit [170]. GM-CSFR signals induce the activation of JAK2 and downstream molecules STAT5 and PI3K [171]. Activation of these pathways induces survival, proliferation and differentiation of innate immune cells [167].

Throughout the differentiation of Th17 cells, IL-1β and IL-23 activate RORγt and promote increased expression of GM-CSF, which is associated with the pathological phenotype of Th17 cells [22, 37]. Studies with the EAE mouse model have shown that GM-CFS-producing Th17 cells are drivers of inflammation, corroborating the pathological phenotype associated with this cytokine [172, 173]. Therefore, GM-CSF has been associated with autoimmune pathologies that display pathogenic Th17 cells, such as MS and RA [174].

1.6.2.5. Interleukin 10 (IL-10)

IL-10 was first known as cytokine synthesis inhibitory factor (CSIF) and initially identified as a cytokine secreted by Th2 cells to regulate the production of IFN-γ by Th1 cells [175]. However, various studies have demonstrated that IL-10 is also produced by B and T cells as well as neutrophils, eosinophils, NK cells and mast cells [176].

IL-10 signals through IL-10R, a receptor composed of two subunits, IL-10R1 and IL-10R2 [177]. IL-10R1 and IL-10R2 intracellular domains are constitutively associates to JAK1 and TYK2 kinases respectively [178]. After activation IL-10R induces the phosphorylation of JAK1 and activates STAT1, STAT3 and STAT5 [178, 179].

IL-10 is an anti-inflammatory cytokine harboring different activities in the B and T cell compartment. For instance, IL-10 induces maturation of B-cell into plasma cells [180]. IL-10 acts on antigen presenting cells to reduce their expression of MHC-II and of IL-12 and then contributes to decrease T-cell activation [181, 182]. In the T cell compartment, IL-10 exhibits different effects upon the cell type. IL-10 enhances the expression of FoxP3 and the suppression capacity of Treg cells [183]. In $CD4^+$ T cells, IL-10 inhibits the phosphorylation of CD28 and induces an anergic state [184].

More, the expression of IL-10 by Th17 cells is associated to a Th17 non-pathogenic function profile. [22]. Interestingly, Zielinski et. al. showed that Th17 cells primed with antigens related to S. aureus, exhibit a transient expression of IL-10 concordant to a decreased in IL-17 production [185]. While, Th17 cells differentiated in presence of IL-6 and TGF-β1 produce IL-10 in addition to their production of IL-17 [73]. The analysis of the Th17 cell kinetic of differentiation by Yosef et al [186] has revealed that IL-10 is expressed at the amplification phase of the differentiation process and play a critical role in the Th17 cell mechanism of self-regulation [186] **(Figure 2)**.

1.7. Th17 cells and autoimmunity

There are approximately 80 known autoimmune diseases, of which half are considered rare pathologies [187]. Autoimmune diseases (AIDs) are chronic, inflammatory pathologies that affect mostly young adults at reproductive age. Autoimmune diseases are diverse, and they can manifest as tissue-specific or as systemic disease. Despite the variability of AIDs, these pathologies have a common etiology: a reactivity to autoantigens by the adaptive immune system [187]. AIDs are multifactorial and arise from a combination of genetic predisposition, sexual dimorphism,

environmental factors (e.g., diet, pollution and infections) and an inadequate control by Treg cells **(Figure 6)** [188]. Moreover, AIDs are pathologies that may arise years after the initial breakdown of the immune tolerance induced by unknown factors [189, 190]. To date, there is no definitive cure for any autoimmune disease, although remission can be achieved. Nevertheless, discovery of the IL-23/Th17 pathway has shed light onto some pathological mechanisms and opened the possibility of new efficient and long-term recovery therapeutic approaches.

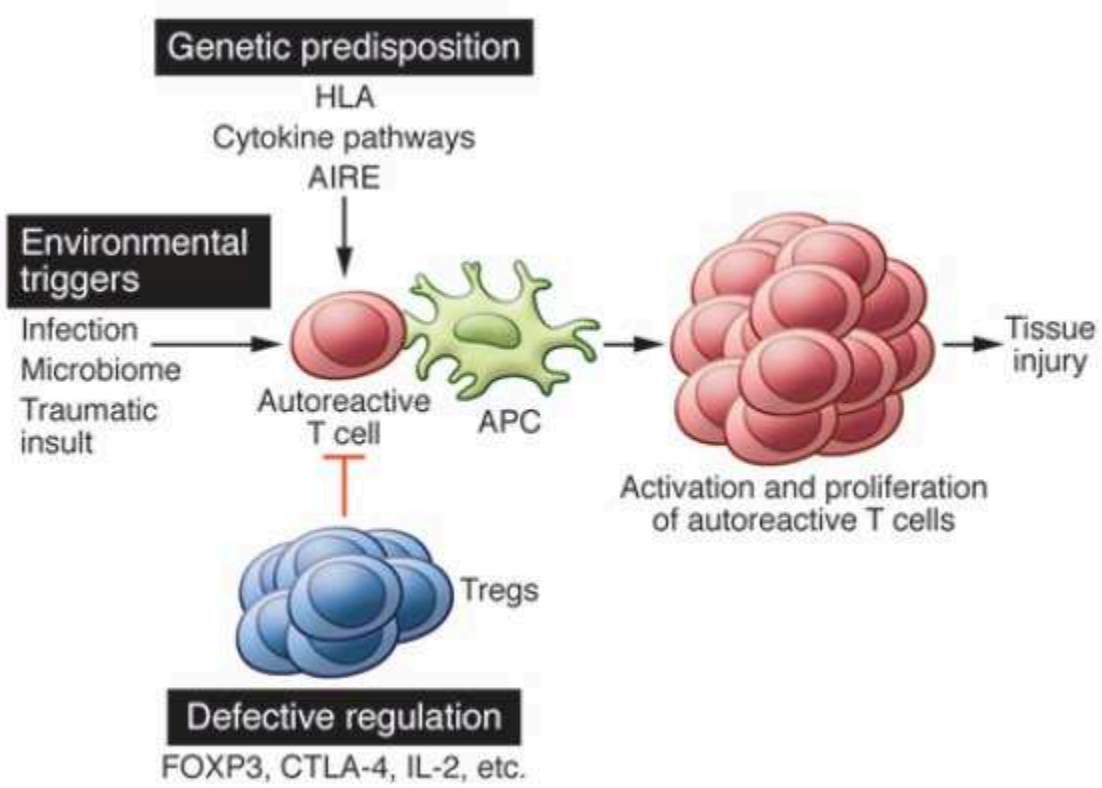

Figure 6 : Autoreactive cells arise from the convergence of different factors.
The hallmark of autoimmune diseases is the presence of autoreactive T-cells. Autoreactive T-cells arise due to a combination of factors, such as A) genetic predisposition in immune-related genes; B) environmental triggers such as infection, changes in diet and microbiota; and C) loss of the regulatory capacity of Treg cells. The conjunction of these factors in an inflammatory microenvironment may promote development of autoreactive T-cells and autoimmunity. [191]

As previously mentioned, the function of Th17 cells is to protect from pathogens at the mucosal barriers such as the intestines and lungs. In these tissues, changes in the homeostasis between commensal bacteria and immune cells might induce a constant inflammation that results in autoimmune diseases. For instance, changes in the microbiota can damage the mucosal barrier of

the gut and promote excessive development of Th17 cells that results in autoimmune pathologies such as Crohn's disease and ulcerative colitis [192]. Although the exact mechanism by which T-cells become auto-reactive is not yet known, data suggest that a loss of tolerance may be induced by an epitope mimicry of microbial peptides or activation of natural occurring autoreactive T-cells by chronic inflammation [193, 194].

Moreover, Th17 cells that developed outside of the mucosal barrier are considered as pathogenic and are associated with inflammatory autoimmune diseases [132, 195]. Indeed, in nonbarrier tissues such as the brain or the joints, the presence of Th17 cells has been linked to the pathological processes of MS and RA [196, 197].

The development of eGC in tissues targeted by autoimmune reactions is a common feature that has been associated with the presence of IL-17^{+} cells and/or Th17 cells. For example, IL-17 has been demonstrated to be essential for the formation of eGC in the BXD2 mouse model, a mouse strain that develop spontaneous erosive arthritis and glomerulonephritis [198]. IL-17 effects included activation of B cells and induction of activation-induced cytidine deaminase to promote B cell somatic hypermutation. Moreover, IL-17 also controls the chemotactic capacity of B-cells by modulating regulators of G-protein signaling (Rgs) 16 and 13 and arrest cells within the eGC [198]. However, a similar report showed that in human B cells, IL-17 downregulates Rgs16 allowing B cells to react to chemokines such as CXCL12 [199]. Moreover, in models of lung infection and chronic inflammation (inducible bronchus-associated lymphoid tissue), IL-17 was demonstrated to be essential for the lymphoid structures' initial development. Although IL-17 can also be produced by lymphoid tissue inducer (LTi) cells, Th17 cells and IL-17 producing Tfh cells were critical for the GC formation in this model [200]. Furthermore, the implication of Th17 cells in eGC formation has been associated with their expression of podoplanin (Pdpn) [201], an anchoring protein involved in the formation of lymphoid tissues [202]. In vitro, differentiation of Th17 cells with IL-6, IL-1β, TGF-β1 and IL-23 induce the expression of Pdpn by Th17 cells that do not secrete IL-17 or IFN-γ [203]. However, in another study, Pdpn expression contribute to stimulate high expression of IL-17 by PBMC when cocultured with synoviocytes of RA patients [204]. In the mouse model of MS, EAE, Th17 cells expressing Pdpn are present in the central nervous system (CNS) and are requires for the eGC formation [201]. Similarly, in arthritic SKG, Th17 cells infiltrated in the mice's joints harbors an overexpression of Pdpn [205]. Thus, expression of IL-17 and Pdpn by Th17 cells are associated to

physio pathogenic events occurring in AIDs. The implication of Th17 cell in specific AIDs is detailed in the following paragraphs.

1.7.1. Th17 cells and Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS. MS is driven by myelin protein-reactive $CD4^+$ T cells against the oligodendrocytes in the CNS [206, 207]. Studies using EAE, the MS mouse model, have shown that IL-6 and IL-23 are essential for the development of clinical symptoms [36, 208]. Moreover, IL-23-induced Th17 cells harbor the ability to cross the blood-brain barrier. Within the CNS, Th17 cells promote the infiltration of B-cells and formation of ectopic germinal centers (eGC) [1, 201]. Furthermore, Th17 cells' production of GM-CSF stimulates antigen-presenting cells (APC) to produce IL-23, creating a positive feedback loop between Th17 cells and APC [173]. Consistent with the observation in EAE, human post-mortem biopsies have demonstrated the presence of eGC with proliferating $CD20^+$ B-cells [209] and an increased expression of IL-17 in the CNS [210]. Observations on PBMC have shown that compared with healthy controls, MS patients harbor a higher production of IL-23 and IL-17 by monocyte-derived dendritic cells (DC) and $CD4^+$ T-cells, respectively [211]. These data imply that Th17 cells are highly implicated in the development of MS.

1.7.2. Th17 cells and rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease in which Th17 cells are also implicated. RA is characterized by synovial inflammation and destruction of joint cartilage and bone. Destruction of these tissues is driven by pathogenic Th17 cells present in the synovium of patients' joints [197]. Predisposition to RA has been associated with polymorphisms on IL23R and PTPN22 [212, 213]. The inflammatory hallmarks in RA synovium are an overexpression of cytokines such as IL-1β, TNFα, IL-17 and, IL-21 and infiltration of T and B cells, macrophages and dendritic cells [17, 214, 215]. This inflammatory cocktail induces the destruction of bone and cartilage. Th17 cells participate in bone erosion through their expression of IL-17 and IL-21. Indeed, IL-17 and IL-21 promote the development of osteoclasts (hematopoietic cells that reabsorb bone) and the production of matrix metalloproteinase 1 and 3 by synovial fibroblasts [216, 217].

Interestingly, RA synovial fluid presents a high concentration of IL-23p19, which has been correlated with higher bone erosion [123]. Moreover, experiments in vitro have shown that IL-17 can induce

the expression of CCL20 in synoviocytes which attracts Th17 cells [215]. In vivo studies with CIA, the mouse model of RA, have shown that lack of IL-23p19, IL-17 or IL-6 protects mice from the development of the pathology [4, 218, 219]. Intriguingly, therapies that target IL-23 or IL-17 do not seem to be effective in treating RA. In contrast, therapeutic monoclonal antibodies that block the effect of IL-6 (tocilizumab and sarilumab) are now approved for treatment of RA patients [220] [221].

1.7.3. Th17 cells and psoriasis

Psoriasis is an inflammatory skin disorder due to a dysregulation between keratinocytes and infiltrated immune cells. Psoriatic lesions (scaly and erythematous plaques) are produced by an excessive proliferation of keratinocytes in the epidermis [222]. Like other AIDs, the origin of psoriasis is unknown. However, studies have shown an association between polymorphisms in HLA, IL-23R and IL-12 and a predisposition to psoriasis [223]. Thus far, four autoantigens have been found in psoriasis: LL-37 (an antimicrobial peptide), ADAMTSL5 (A disintegrin and metalloprotease domain-containing thrombospondin type-1 motif-like 5), keratin 17, and lipid Ags generated by phospholipase A2 group IV D (PLA2G4D) [224]. Moreover, psoriasis is now accepted as a pathology induced by IL-23-stimulated Th17 cells. Indeed, psoriatic skin lesions present an increased number of DC that overexpress IL-23p19 [121]. Thus, excessive production of IL-23 in psoriatic skin promotes the differentiation of pathogenic Th17 cells and expression of IL-17 and IL-22. In the skin, IL-17 and IL-22 stimulate keratinocytes to express cytokines and chemokines. For instance, IL-17 induces the expression of IL-8, CCL1, CCL3, CCL5 and, notably, CCL20, which recruits $CCR6^{+}$, Th 17 cells and other innate immune cells [225, 226]. IL-17 can stimulate keratocytes and promote the expression of pro-inflammatory cytokines, including IL-6 and IL-8 [226]. Interestingly, abnormal proliferation of keratinocytes has been shown to be induced by IL-22 [227, 228]. Of note, the implication of the IL-23/Th17 pathway in psoriasis has been confirmed by the good results shown by clinical trials of monoclonal antibodies that target this pathway and led to recent approvals of ustekinumab (anti-IL-12/IL-23) and guselkumab (anti-IL-23p19) to treat psoriasis.

1.8. Therapy targeting of Th17 cells

Studies showing the relevance of the IL-23/Th17 pathway in AIDs have opened new therapeutic possibilities. Relevant therapies that target Th17 cells have been approach in two different ways: **1)**

through biologically active monoclonal antibodies that specifically target Th17 cell-related cytokines or **2)** through small compounds that target Th17 cell transcription factors.

1.8.1. Monoclonal antibodies

The first monoclonal antibody approved by the U.S. Food and Drug Administration was Muromonab in 1986 [229]. Muromonab, an anti-CD3 antibody, was a murine IgG2a used to treat acute allograft rejections. This antibody was produced by hybridoma in pathogen-free standard-bred mice [230]. Therefore, this antibody presented high immunogenicity and behaved as a foreign antigen, inducing many side effects [230]. However, new biotechnological advances allowed the development of monoclonal antibodies with few or no murine components in their structure **(Figure 7)**, which makes them less immunogenic and therefore more effective for treating pathologies [231]. Additionally, a nomenclature has emerged with monoclonal antibodies named depending on their origin and the human representative composition (murine, chimeric, humanized or fully humanized) **(Figure 7).**

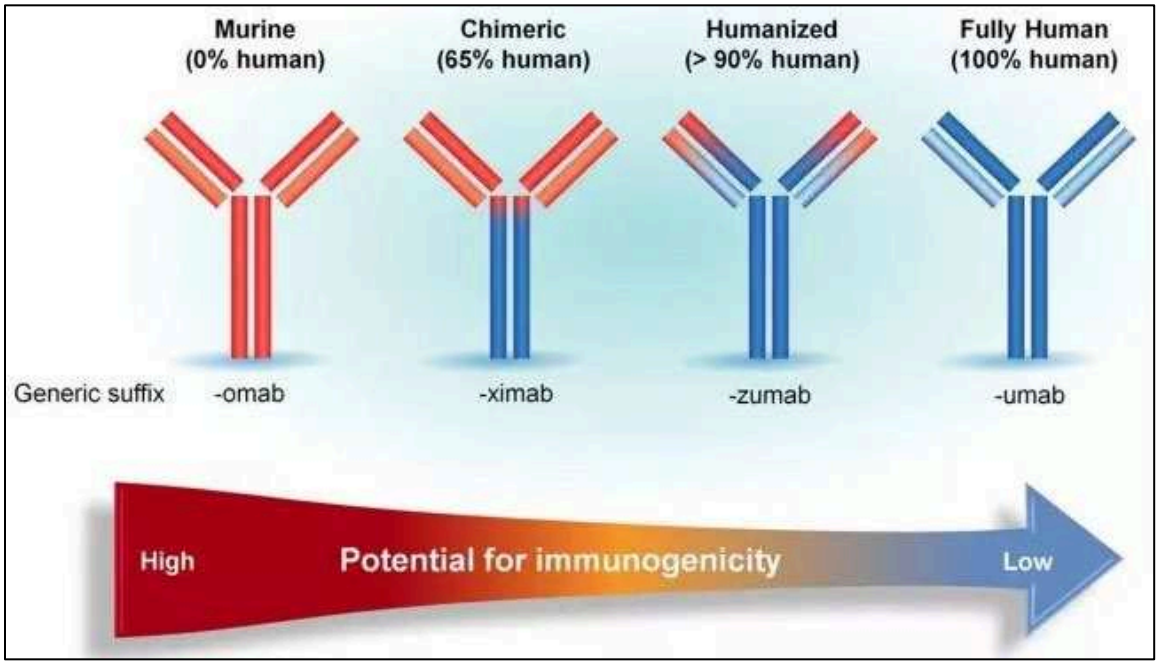

Figure 7 : Structures of monoclonal antibodies and their potential immunogenicity
Therapies with monoclonal antibodies are on the rise. The main characteristic of monoclonal antibodies is their specificity. Advances in biotechnology have allowed us to overcome their immunogenicity potential and to develop fully human monoclonal antibodies to treat autoimmune diseases. http://www.icartab.com/article/1/3.html.

Monoclonal antibodies can exert their therapeutic action by either neutralization, complement activation or stimulation of cell-mediated cytotoxic activities [232]. Due to the high impact of Th17-related cytokines, therapeutic monoclonal antibodies have been focalized on the neutralization of the cytokines related to Th17 cells. For instance, secukinumab and ixekinumab are monoclonal antibodies that neutralize the biological activity of IL-17A. These monoclonal antibodies have been shown to be effective in the treatment of psoriasis with minor negative effects [233]. In contrast, Crohn's disease patients showed no beneficial effects and disease worsening [234]. Moreover, clinical trials for Crohn's disease have shown similar results by targeting IL-17R with brodalumab [235]. The deleterious effects of anti-IL-17A and anti-IL-17R targeted treatments in Crohn's disease have been associated with the essential function of IL-17A in the intestine as a protective cytokine.

Nevertheless, IL-17A production by Th17 cells can also be indirectly targeted by the neutralization of cytokines involved in Th17 cell differentiation. Ustekinumab is a monoclonal antibody that targets IL-12p40 (one of the two IL-23 subunits) and blocks the activity of both IL-12 and IL-23. Ustekinumab is now used for the treatment of psoriasis and Crohn's disease, and patients display a significant reduction of the Psoriasis Area and Severity Index (PASI 75) and a significantly reduced endoscopic disease activity, respectively [124, 125]. Ustekinumab has also shown encouraging results in a phase II clinical trial for the treatment of SLE [236]. In other AIDs such as MS, however, ustekinumab has not ameliorated patients suffering, probably due to the low therapeutic antibody penetration into the CNS, the blood-brain barrier being a limiting factor [237]. Similarly, RA patients treated with anti-IL-23/23 showed no signs of improvement. This unexpected result could raise the question of the reduced implication of Th17 cells in already well-established RA disease [238].

Monoclonal antibodies that target the IL-23p19 subunit (i.e., tildrakizumab, guselkumab, risankizumab and brazikumab) have also shown different outcomes in different autoimmune pathologies. For instance, guselkumab has been recently approved for treatment of moderate to severe psoriasis plaque [239, 240]. In contrast, guselkumab has not shown efficacy in a phase II clinical trial for the treatment of RA [238]. Clinical trials of risankizumab and brazikumab for treatment of IBD are still in progress (NCT03398135, NCT03105128, NCT03616821 & NCT03759288). These studies illustrate the active clinical research targeting IL-23 compounds to improve patient outcomes.

IL-6 is a key cytokine in the initial development of Th17 cells and plays a critical role in the balance between Treg and Th17 cells. Antibodies that target the two receptors of IL-6 are already available

for the treatment of RA (tocilizumab and sarilumab) [220, 221]. Antibodies that target IL-6 directly are also under investigation in clinical trials to prove their effectiveness against RA [241].

1.8.2. Small chemical molecules

Inhibition of Th17 cells can also be achieved with small compounds that interfere with RORγ or with the JAK/STAT pathway. Compared to biological treatments, these compounds present the advantage of acting intracellularly.

Inhibitors of RORγt such as digoxin, ursolic acid and other compounds such as VTP-43742, TMP778, TMP920 and GSK2981278 **(Figure 8)** are potential therapeutic candidates for Th17-associated autoimmune diseases [242-245]. These compounds act either by preventing RORγ DNA binding or by disrupting the transcriptional regulation following the RORγ-DNA binding [245]. So far, many studies have shown that these molecules effectively control Th17 cell effects in murine models of MS [245, 246] and RA [243] and in autoimmune uveitis [247]. However, the single clinical trial using such a strategy has failed to show effectiveness of treating psoriasis with the GSK2981278 molecule [248].

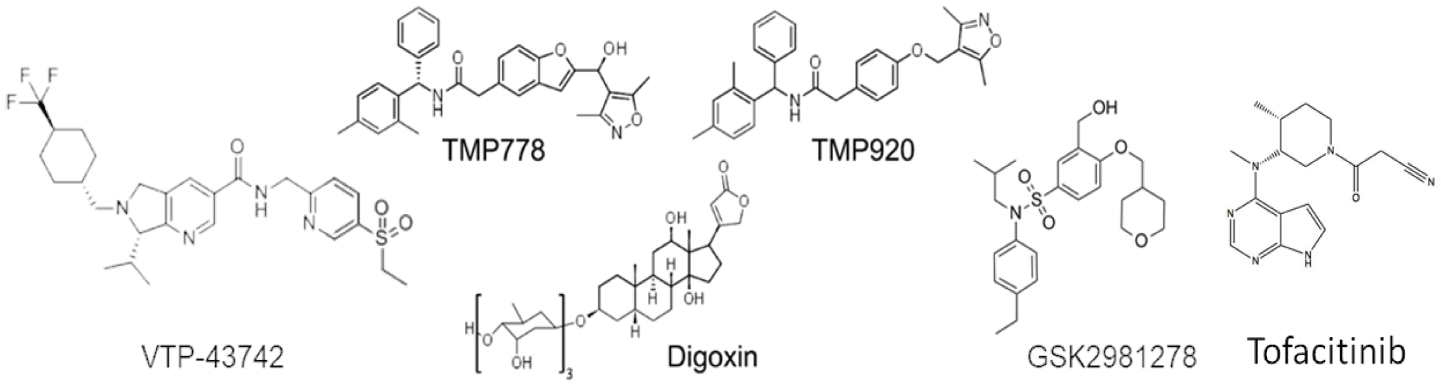

Figure 8 : Chemical compounds that target Th17 cells

Chemical compounds are being test as potential therapies in AIDs. The ability of these compounds to act intracellularly make it possible to target transcription factors such as RORγ and STAT3 to control the differentiation of pathogenic T-cells. [245].

The implication of the JAK/STAT pathway in the differentiation of Th17 cells offers other therapeutic targets. Tofacitinib is a reversible JAK inhibitor that blocks the phosphorylation and activation of all

JAK family members [249]. This synthetic molecule has been tested for the treatment of RA and has been shown to be effective for patients, providing clinical improvement superior to that of methotrexate-treated patients [250]. Moreover, another clinical trial also showed that tofacitinib induced a significant improvement in patients affected with psoriasis [251].

Globally, the Th17 cell presents various therapeutic targets, from its intracellular transcription factors to IL-17, its cognate cytokine, and the upstream cytokines required for its development **(Figure 9)**. The diverse results, obtained in different pathologies, illustrate that therapeutic targets can be achieved at different levels to ameliorate autoimmune pathologies.

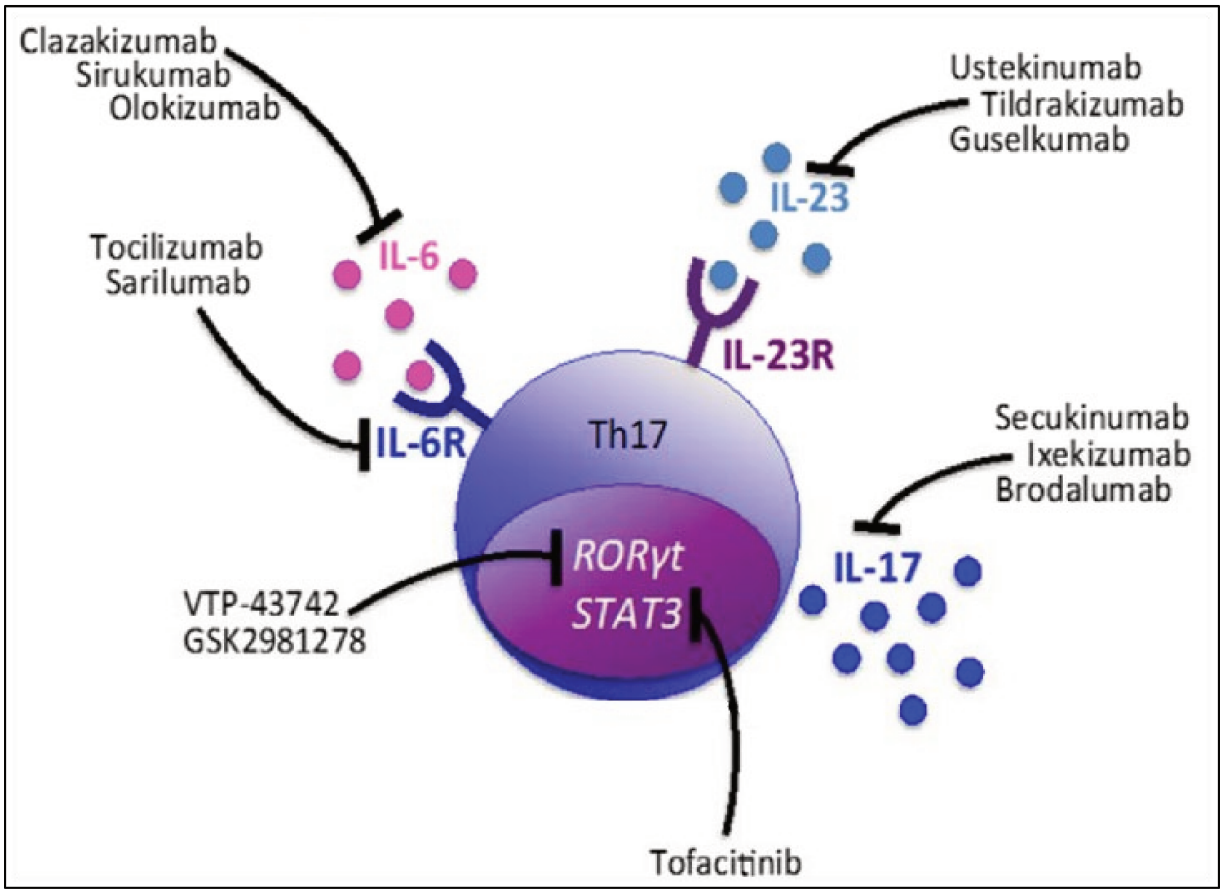

Figure 9 : Potential therapeutic targets in Th17 cells

Th17 cells are drivers of autoimmunity. Therapeutic approaches with monoclonal antibodies aim to block the effect of Th17 cell-related cytokines or Th17 receptors, while chemical compounds aim to disturb differentiation of Th17 cells through the inhibition of Th17 cell transcription factors. Most of these molecules are currently in clinical trials for treatment of AID. [244]

2. Myasthenia gravis (MG)

Autoimmune myasthenia gravis (MG) is a chronic inflammatory disease that induces fatigability and muscle weakness after continuous effort. Myasthenic symptoms are due to the presence of autoantibodies that attack proteins present at the postsynaptic membrane on striated muscle, including the acetylcholine receptor (AChR), the muscle-specific kinase (MuSK), the lipoprotein related protein (LPR4), and agrin [252, 253]. MG is a rare spontaneous and complex pathology that is classified with respect to the type of antibodies present, the affected tissues and the age of the patients [254].

2.1. Historical aspects of myasthenia gravis

The first descriptions of what we call now myasthenia gravis were recorded in the 17th century. Thomas Willis described cases of patients who were capable of doing all their activities in the morning but were completely unable to do them at noon. His explanation was that patients had a circulating substance that induced paresis as a function of its concentration [255, 256].

In the 19th century, Wilhelm Erb described the pathology of patients as unusual, special, peculiar, or strange. However, he also remarked that patients presented bilateral ptosis, diplopia facial paresis and neck weakness; this would be one of the first medical descriptions of MG symptoms [255]. Throughout the 19th century, some reports of the pathology were published, and in 1893, Samuel Goldflam reviewed published papers and made a description of a myasthenia patient's symptoms, severity and prognosis [255]. Of note, at this point the pathology had no specific name, and for some time it was known as "Erb's disease" or "Erb-Goldflam disease" [256]. It was in 1895 that Frederich Jolly came up with the name "*Myasthenia gravis pseudo-paralityca"* to describe two of its patients [255, 256].

In 1901, at an annual convention in Germany, Carl Weigert reported an association between myasthenia and the thymus. He had observed a thymic tumor (thymoma) in a deceased myasthenic patient [257]. This brought attention to the thymus of MG patients. Subsequent reports determined that thymic hyperplasia was a common feature in MG patients [258]. These observations opened the possibility of considering thymectomy as a possible therapy. Meanwhile, in 1934 Dr. Walker reported that physostigmine (a cholinesterase inhibitor) induced a considerable improvement of clinical symptoms in MG patients, and in 1935 Blake Pritchard corroborated the beneficial effect of

a cholinesterase inhibitor (prostigmin) and demonstrated the impact in the transduction of the electrical impulse between the nerve and the muscle [259].

In 1962, the first description of the blocking effect of α-bungarotoxin on neuromuscular transmission was published by Chang and Lee [260]. This observation led other researchers to study the binding site of α-bungarotoxin to finally determine that it acts on the same site or close to the binding site of acetylcholine and to therefore quantify the AChR in muscle [261]. Then, in 1973, a study showed that MG patients presented with a reduction in the number of AChR [262]. In the same year, Patrick and Lindstrom injected AChR (obtained from the electric organ of electric eel) in rabbits to induce an immune reaction and observed that, after a second injection of AChR, animals developed the clinicals symptoms of MG patients [263]. These results established the basis to categorize MG as an autoimmune disease. One year later, Almon and colleagues demonstrated that serum from myasthenia gravis had globulins capable of interacting with AChR and blocking the effect of α-bungarotoxin [264]. In 1975, Toyka showed that passive transfer of IgG from MG patients to mice reduced the number of AChR in the animals and decreased the response of their muscles to nerve stimulation [265]. Since it was clear by this time that MG patients presented anti-AChR antibodies, in 1976 the first report came showing that plasma exchange had a beneficial effect in MG patients [266].

Despite the advanced understanding of MG, there were still blanks to fill. For instance, anti-AChR antibodies were not present in all MG cases [267]. It was not until the 2000s that antibodies against muscle-specific kinase (anti-MuSK) and lipoprotein receptor-related protein-4 (anti-LRP4) were implicated as antibodies capable of inducing MG [268]. To date, research in MG is mainly focused on understanding the physiopathological mechanism behind the presence of antibodies and on looking for more effective therapies.

2.2. Epidemiology

Among MG patients, 80% to 85% harbor antibodies against AChR, 4% have anti-MuSK antibodies and less than 2% have antibodies against LRP4 [269-271]. The remaining 10–15% of MG patients are seronegative to the identified target proteins, suggesting the probability that new protein targets are still to be discovered [272].

The incidence rate of all types of MG range between 1.7 and 21.3 cases per million persons per year [273]. Epidemiological studies have showed that the prevalence of MG patients is between 15 and

179 cases per million persons. Myasthenia gravis follows a bimodal age tendency, with a first peak around the age of 30 years that is known as early onset myasthenia gravis (EOMG). The second peak occurs after the age of 50 years and is known as late onset myasthenia gravis (LOMG) [269, 270].

2.3. Classification of autoimmune myasthenia gravis

2.3.1. Ocular form of myasthenia gravis

The first manifestations of MG are diplopia (double vision), unilateral ptosis (dropping of the upper eyelid), dysphonia (voice disorders) and neck weakness [254]. Ocular manifestation occurs in most MG patients. However, in 10–15% of patients, symptoms remains restricted to ocular manifestations and will not develop to a generalized form of MG [270]. In this group, about one-half of the patients do not present anti-AChR antibodies. Thymoma is not a common feature in this group [274].

2.3.2. Generalized form of myasthenia gravis

While a small proportion of MG patients develop only an ocular form of MG, in most patients there is a progression to the bulbar and limb muscles and thus a developing of a generalized form of MG in the 2 years following the appearance of the ocular symptoms. This means that common activities such as walking and exercising are affected by this disorder [270]. Moreover, MG can be life-threating due to respiratory failure that may occur in patients during a myasthenic crisis [254]. But the MG mortality rate is low—between 0.06 and 0.89 per million persons per year [273]. The classification of autoimmune MG is based on the presence of antibodies, onset age, and clinical symptoms.

2.3.3. Myasthenia gravis with anti-AChR antibodies (MG AChR^{+})

MG patients with antibodies against acetylcholine receptor are the largest group of MG patients. They account for 80% of all MG patients [254]. This form affects mainly young women [254]. The diagnosis is confirmed based on the detection of anti-AChR antibodies in the serum.

In AChR$^+$ MG patients, the most common subclasses of anti-AChR antibodies are IgG1 and IgG3, subtypes that activate the complement system [275]. Thus far, three mechanisms of action of the antibodies have been proposed: **1)** the degradation of AChR, **2)** the internalization and proteolysis of AChR and **3)** the activation of the complement at the neuromuscular junction [276-279]. In addition, IgG1 and IgG3 are able to crosslink antigens, therefore inducing an active internalization of the clustered AChR [280, 281].

An intriguing feature of AChR$^+$ MG patients is that the pathology severity is not correlated with the concentration of circulating anti-AChR antibodies [267]. This is probably due to posttranslational modifications of autoantibodies (i.e., sialylation and glycosylation), which can be introduced by antibody-producing B-cells [282, 283].

MG AChR$^+$ patients display a neuromuscular disorder that results from either thymic hyperplasia or thymoma. Hyperplastic thymuses are characterized by abnormal infiltration of B-cells that cooperate with T-cells to set-up eGC. Interestingly, in this group of patients, the antibody concentration correlates with the degree of thymic hyperplasia [284]. Thymoma is a thymic tumor caused by an abnormal development of epithelial cells and is usually present in LOMG patients with anti-AChR antibodies.

Since the characteristics of AChR$^+$ MG vary upon the age and the disease symptomatology, some subclassification has been proposed, as presented next.

2.3.3.1. Juvenile onset myasthenia gravis (JMG)

Juvenile myasthenia gravis (JMG) patients present MG symptoms during the childhood and the adolescence period (before 18 years old) [285] [286]. JMG is characterized by thymic hyperplasia and in some cases thymomas [285, 287], and it accounts for 10–15% of all cases of MG [288]. Of note, among the prepubertal and post-pubertal MG patients, 30–50% of JMG patients are seronegative. The general treatment of JMG patients is the same as adult MG patients (anticholinesterase, glucocorticoids and immunosuppressors). These patients are eligible for thymectomy [289].

2.3.3.2. Early onset myasthenia gravis (EOMG)

The EOMG patients present the first symptoms of MG before they are 50 years old. This group of patients present thymic hyperplasia with eGC [290]. Within this group, females are more affected than males by a ratio of 4:1 and are the most suitable patients to undergo thymectomy [254, 291].

2.3.3.3. Late onset myasthenia gravis (LOMG)

LOMG accounts for a group of patients who present the first symptoms after 50 years of age. LOMG patients often present a thymoma (cancer of thymus). In patients without thymoma, thymectomy is less effective than for EOMG [292]. LOMG patients with thymoma usually present anti-AChR antibodies. However, this specific group often develops additional autoantibodies against other proteins of the muscle such as anti-titin, anti-ryanodine or anti-striated muscle [293].

2.3.3.4. Low-affinity AChR$^+$ MG

Low-affinity AChR$^+$ MG is relatively rare (about 5%). This group presents symptoms to similar those of AChR$^+$ patients but without detectable antibodies against AChR. However, a study in 2008 by Leite et al. demonstrated that these patients do harbor low-affinity anti-AChR antibodies. These antibodies are not detectable by conventional assays [294] but are able to bind the rapsyn-clustered AChR and to fix the complement [294]. Of note, in this group of patients, the thymus presents changes similar to those seen in EOMG AChR$^+$ patients. The low-affinity AChR$^+$ patients can benefit from classical treatments like acetylcholinesterase inhibitors, plasma exchange therapy and immunosuppressive therapy [295].

2.3.4. Myasthenia gravis without anti-AChR antibodies

2.3.4.1. MG with anti-muscle-specific kinase (MuSK) antibodies

MuSK$^+$ MG patients account for 4% of all MG cases and 40% of anti-AChR negative patients with generalized myasthenic symptoms [268]. MuSK$^+$ MG affects mostly middle-aged women [296] with symptoms including facial, bulbar and respiratory muscle weaknesses. In contrast to AChR$^+$ patients, MuSK$^+$ patients do not present morphological and anatomical thymic changes [297]. Anti-MuSK antibodies are mostly IgG4 and do not activate complement [298]. The mechanism of action of anti-

MuSK antibodies is most likely disturbing agrin-MuSK-AChR clusters and their distribution during synapse formation, leading to muscular atrophy [268].

2.3.4.2. MG with anti-low-density lipoprotein receptor related protein 4 antibodies (LRP4)

Myasthenia patients with anti-LRP4 antibodies represent around 20% of MG patients with generalized MG and without either AChR or MuSK antibodies. LRP4 is the receptor for agrin released by the terminal nerve at the neuromuscular junction (NMJ) [299]. LRP4 participates in the clustering of AChR by activating MuSK [300, 301]. The features of this MG form are the presence of anti-LRP4 antibodies, mostly IgG1 subtype, unaltered thymus and clinical symptoms similar to those of $AChR^{+}$ patients [269, 302, 303].

2.4. Etiology

Myasthenia gravis is a multifactorial pathology. The trigger factor of which is still unknown. While numerous studies have determined the impact and involvement of genetic and gender predisposing factors [291, 304-307], the MG etiology remains unknown, even though environmental factors, such as diet/microbiota or virus exposure have been suggested [308-312].

2.4.1. Genetic predisposition

It is well known that autoimmune myasthenia gravis is not a hereditary pathology. However, as with many other autoimmune diseases, gene polymorphism may predispose some to the development of the pathology. The major histocompatibility complex (MHC) is the most important gene in the genome related to the immune system and most autoimmune diseases. The haplotype HLA A1-B8-DR3 displays a strong association with EOMG patients [313, 314]. In North American and Italian cohorts, CTL4 and HLA-DQA1 variants are associated with MG [306]. Moreover, CTL4 has also been associated with MG in the Swedish population [315].

In EOMG patients, a polymorphism in the promoter of CHRNA1, the gene coding for α-AChR, is associated to MG in the European population [316]. Additionally in the European population, specific haplotypes for the B-cell activator factor (BAFF) and for VAV1 guanine nucleotide exchange factor are associated to MG development [317].

Polymorphisms in cytokines such as IFN-γ, IL-10 and IL-12 have also been found in MG patients in a Turkish population [318], and TNFα polymorphisms have been associated in MG female Swedish patients [319] and in MG with thymoma in a Chinese cohort [320]. T-cell activation genes such as PTPN22 also present polymorphisms associated with MG patients in a French cohort [321]. Interestingly, a gain-of-function polymorphism in this gene was related to MG patients with thymoma [322].

Despite the numerous genetic studies that have shown the relationship between gene polymorphisms and susceptibility to developing MG in different populations, MG cannot be defined by genetic factors alone, and other factors may have a role in the pathology development.

2.4.2. Gender influence

Sex hormones are a factor that contribute to the gender bias in autoimmune diseases. Myasthenia gravis is no exception, as in EOMG females represent around 70% of the patients [273]. Estrogens are the primary female sex hormone and signal through the estrogen receptors α and β (ERα and ERβ). In MG patients, we have demonstrated that there is an increased expression of ERα in thymic T-cells [323], and that stimulation of medullar thymic epithelial cells (mTEC) with estrogens downregulates the expression of α-AChR and MHC-II, inducing a possible gender-defective tolerization to AChR [291].

2.4.3. Environment

2.4.3.1. Virus and activation of IFN pathway

Virus involvement in the induction of MG has been proposed by different groups [310, 324, 325]. Studies have shown evidence of thymic exposure to viruses such as poliovirus [325] and Epstein-Barr virus [326]. Moreover, infiltrated thymic B-cells overexpress TLR7 and TLR9, especially—although not exclusively—those in the eGC [327]. Toll-like receptors are known to activate the IFN-I pathway. The MG thymus shows an overexpression of TLR3 and PKR as well as interferon regulatory factors that indicate an antiviral response [324]. Indeed, in highly hyperplastic MG thymuses, an upregulation of IFN-α and IFN-γ has been reported [328, 329].

2.4.3.2. Microbiota

Diet might change and alter the equilibrium of the intestinal microbiota. Evidence demonstrates that intestinal microbiota influences functions of the immune system [330]. Recently, two studies analyzed the microbiota of MG patients and reported an increased proportion of *Bacteroidetes* and proteobacteria and a reduction of *Firmicutes*, *Bifidobacterium*, *Aubacterium* and *Clostridium* in MG patients compared with healthy controls [308] [309]. These studies showed a reduced microbial diversity in MG patients, as also observed in other AIDs [196, 331, 332].

Along this line, there are reports of probiotic treatment in the EAMG model. Probiotics are live microorganisms that may confer a health benefit to the host. In the EAMG model, rats receiving probiotics displayed reduced clinical manifestations [333] [334]. In addition, $CD4^+$ T-cell of probiotic-treated rats had a reduced production of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-6 and IL-17A [333]. Of note, the probiotic treatment included five microorganism strains: *Streptococcus thermophilus*, *Lactobacillus reuteri*, *Bifidobacterium bifidium*, *Latobacillus acidophilus* and *Latobacillus casei*. Of those, *Lactobacilli* and *Bifidobacterium* have also shown a beneficial effect in the animal models of IBD and RA [335] [336]. Moreover, Consonni et al. showed that lactobacilli and bifidobacteria could independently ameliorate clinical symptoms in EAMG rats and reduced the production of anti-AChR antibodies [334]. Thus, more studies in MG patients should be carried out to determine the potential therapeutic effect of probiotics.

2.4.3.3. Sun/vitamin D

Vitamin D is involved in diverse functions in the body. Some of the main functions are calcium absorption, osteoclastic maturation and mineralization of collagen for bone formation. Vitamin D effects are transduced by the vitamin D receptor (VDR). VDR activation stimulates, among different effects, a decrease in IL-17 production and a higher activation of Treg cells [337, 338]. MG patients harbor a significant decrease in circulating vitamin D [312, 339, 340]. Interestingly, a pilot study was carried on to supplement MG patients with vitamin D. The results showed an amelioration in only 5 out of 13 patients, with no change in the MGC score in 7 patients and a slight worsening in 1 patient [312, 339]. Therefore, more studies should be carried out to determine the real impact of vitamin D deficiency on MG and specifically on the Treg and Th17 cell balance.

2.4.4. Medicine

Pharmacological drugs side effects can induce autoimmune diseases. D-penicillamine, is a drug used for the treatment of RA, scleroderma and cystinuria [341]. D-penicillamine can induce as a side effect a transitory MG. Indeed, drug-induced MG occurs in 1% of patients and is no different from autoimmune MG, as patients present anti-AChR antibodies and fatigue. However, drug-induced MG stops when treatment with D-penicillamine is discontinued [342]. Interestingly, the mechanism by which D-penicillamine induces MG may rely on the activation of Th17 cells [343]. Biological drugs have also been shown to induce MG. Anti-TNF-α treatment for rheumatic diseases and other autoimmune diseases such as psoriasis and IBD displays side effects such as induction of other autoimmune diseases. There are reports of patients treated with anti-TNF-α for RA and psoriatic arthritis who develop MG [344, 345]. Recently, cancer treatment has focused on immune checkpoints inhibitors. These biologic monoclonal antibodies inhibit T-cell binding of CTLA4 and PD-1 to their receptors and allow T-cell activation and attack on cancer cells. Although these therapies are beneficial for treating cancer, they can also induce autoimmune disorders, including MG. Induced MG in patients treated with anti PD-1 can range from an ocular form of MG to patients with myasthenic crisis [346, 347].

2.5. Physiology of the tissues implicated in AChR$^+$ MG

2.5.1. Thymus

2.5.1.1. Physiological thymus

The thymus is an organ found in the anterior superior mediastinum, in front of the heart and behind the sternum. The principal function of the thymus is to establish the T-Cell maturation and repertoire selection [348]. Of note, 70 to 80% of the circulating lymphocytes are T-cells. The thymus is composed mainly of thymocytes (T-cells in development) and thymic epithelial cells (TEC). Other less representative cells present in the thymus are macrophages, dendritic cells, fibroblasts and myoid cells [349, 350]. The thymus is organized in two main anatomical sections, the cortex and the medulla. Migration of T-cells through the thymic cortex and medulla allows interaction between developing thymocytes and the TEC to obtain mature naïve T-cells after positive and negative

selections [348, 351]. The T-cell journey has been estimated to last around 30 to 35 days in the thymus [352] and 95 % of thymocytes will be eliminated throughout the different steps of differentiation within the thymus [353].

2.5.1.1.1. Thymocytes

Lymphoid progenitor cells originated from the bone marrow colonized the thymus as early thymic progenitors (ETP). The thymic colonization is a four steps mechanism including the progenitor rolling, activation, adhesion and diapedesis [354]. The rolling step occurred though a low interaction of the lymphoid progenitor cells expressing P-selectin glycoprotein ligand 1 (PSGL-1) with the receptor P-selectin expressed by thymic endothelial cells [355]. In addition, chemoattractant molecules (CCL19/21/25) that bind to CCR7 and CCR9 expressed by the progenitor cells contribute to the activation of conformational changes in the membrane integrins expressed by the progenitor cells [356]. Therefore, the integrins bind the receptors ICAM-1 and VCAM-1 expressed by thymic endothelial cells, allowing a strong cell adhesion and easing the progenitor cells diapedesis into the thymus [355]. Of note, thymic colonization is modulated by cellular feedback orchestrated by the progenitor cells present in the niches located inside the thymus [357]. The niches become vacant when the progenitors further differentiate, inducing then periodic waves of progenitor entry inside the thymus. Various teams have aimed to identify and to characterize the cell progenitors that colonized the human thymus [358-362]. So far, no consensus has emerged and controversies on the cellular and molecular markers and processes remain. Nevertheless, the cell commitment to the T-cell lineage encompasses various stages of differentiation characterized by the expression of receptor of cluster of differentiation (CD3, CD4, CD8) (**Figure 10**).

T-cells experience random T-cell receptor (TCR) gene rearrangement, a crucial mechanism to build a library of T cells harboring a wide αβ TCR repertoire [363]. Then such cells may recognize peptide though MHC presentation and be tolerant to self-proteins [363]. [364]. This process may be divided into 4 main steps of T-cell maturation from the thymic cortex to the medulla: 1- T-cell precursor double negative, 2-Double positive expression, 3-positive selection and 4-negative selection.

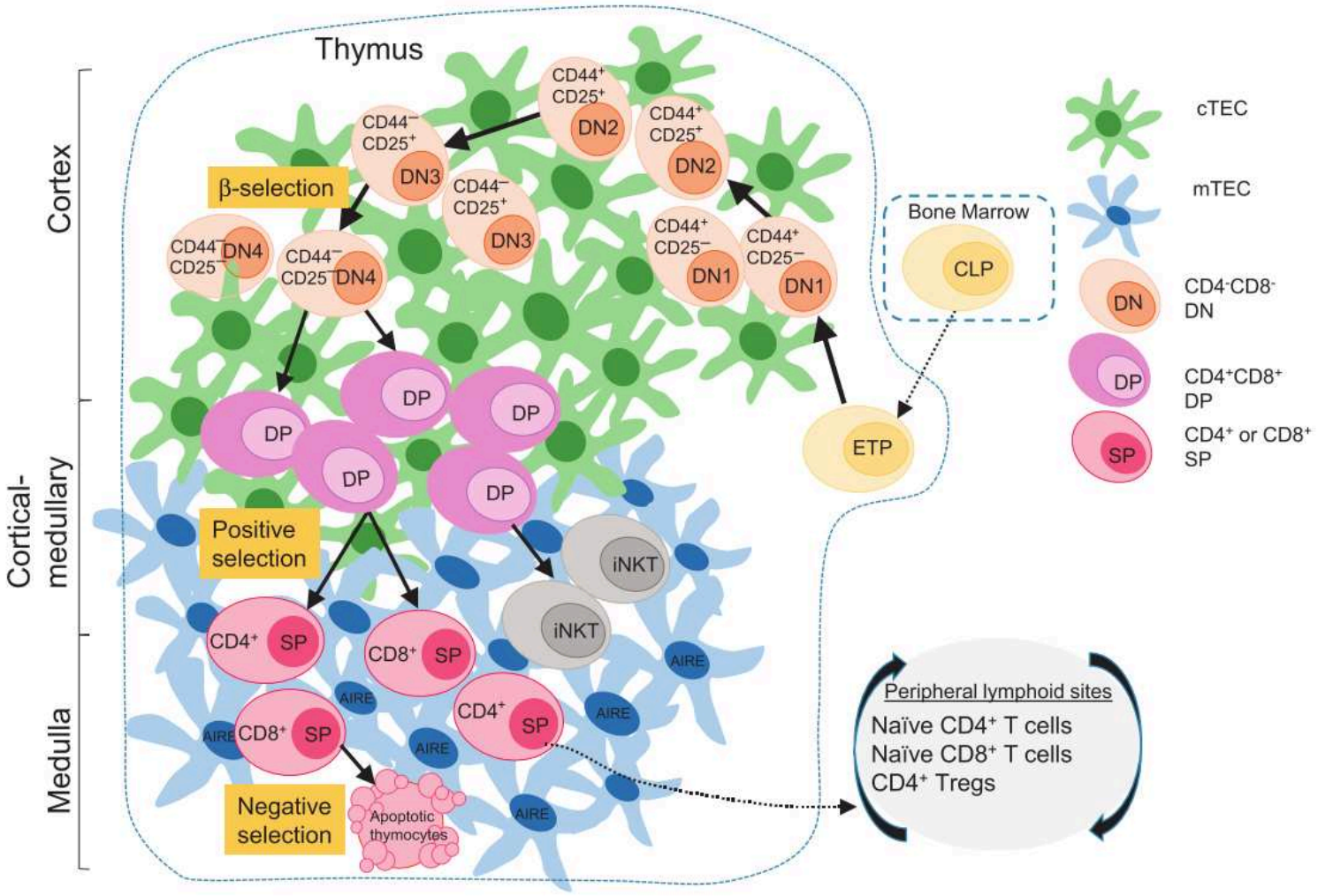

Figure 10 : Positive and negative selection of T-cells in the thymus

The thymus is the organ where central tolerance is established. Thymocytes arrive at the cortico-medullar junction of the thymus with a double-negative phenotype ($CD4^-CD8^-$, DN). Thymocytes then migrate to the outer cortical zone where they acquire a double-positive phenotype ($CD4^+CD8^+$, DP). In this stage, cTEC present MHC-peptides complexes to thymocytes, and those with a moderate affinity are positively selected and continue their differentiation. Positively selected thymocytes then migrate to thymic medulla and acquire a single-positive phenotype ($CD4^+CD8^-$ or $CD4^-CD8^+$; SP). In the cortex, tissue-specific antigens are presented to SP thymocytes by mTEC and DC. Thymocytes with a high avidity for MHC-self peptides are negatively selected, and those with low affinity egress the thymus as naïve T-cells [365].

The differentiation process starts with T-cell precursor migration into the thymus at the cortico-medullary junction following chemoattraction by CCL21, CXCL12 and CCL25 [366]. T-cell progenitors arrive at the thymus as $CD4^-CD8^-$ double-negative cells (DN). Then cells follow their course in the cortex, where they continue their maturation process to get a double-positive phenotype $CD4^+CD8^+$. At this stage, thymocytes undergo positive selection by cTEC [367]. Positive selection of thymocytes

is based on the interaction between the recently expressed TCR on DP thymocytes and peptide-MHC complexes expressed in cTEC. Thymocytes that react with high avidity to the peptide-MHC complex are deleted, while those with low avidity continue with their differentiation process and migration to the thymic medulla in response to CCR7 signaling [348, 367]. Selected thymocytes then are induced to change their phenotype to become single-positive (SP) $CD4^+CD8^-$ or $CD4^-CD8^+$ and go through negative selection. This process is based in the elimination of T-cells that have TCRs with a strong avidity to MHC self-peptides presented by mTEC and dendritic cells. T-cells that survive negative selection will then be exported to circulation as SP $CD4^+$ or $CD8^+$ naïve T-cells **(Figure 10)**.

The last step of thymic differentiation of CD4 and CD8 sp T-cells has been estimated to last 4 to 6 days and included 1-2 cell divisions [368, 369]. The ratio in which CD4 and CD8 T cells emerge from the thymus to the periphery may be estimated to 2.4-4.8 for CD4 to 1 CD8 [352].

Moreover, CD4 and CD8 sp T-cells, once in the periphery as recent thymic emigrant (RTE), may continue to develop and achieve maturation induced by type I interferons, TNF, or CD70 through activation of the NF-*κ*B signaling, a processes that may have been initiated in the thymus [370]. Interleukin 7 receptor is a gene target of the NF-*κ*B signaling pathway in newly developed T-cells [371]; its expression is important for the exported naive T-cells survival [371, 372].

The thymus undergoes involution as a normal physiological aging process. This process may be accelerated at the puberty period to finish at around 25 years of age. The aging thymus encompasses a replacement of the epithelial cells by adipose cells probably originated from thymic stem cells. The mechanisms underlying this process are still not well understood and remain controversial, but various groups have described the process as the result of a multi-factorial combination that includes age-related changes (such as changes in sex hormone) and changes in the thymic microenvironment (including senescent T-cell accumulation, T-cell signals, TCR diversity, and TEC stem cell decrease after birth) [373]. As a consequence, and probably combined with other undefined factors [374], aging contribute to ease a decrease number of mature T-cells emigrate from the thymus to the periphery. Analysis of T cell receptor excision circle that indicate rearrangement of the T cell receptor is a way to estimate the RTE in the circulation as also a mirror of the thymus functionality [375]. Whether age-related decline in TREC frequencies is undeniable, TREC level in aged individuals (thymic functionality) remains to a minimum level even though the thymus displays a significant involution [376].

2.5.1.1.2. Thymic epithelial cells and dendritic cells

Thymic epithelial cells are one of the main components of the thymic microenvironment and are essential for T-cell proliferation, differentiation and repertoire selection. These two populations of TEC, cTEC and mTEC, emerge from a bipotent thymic epithelial progenitor (TEPC). cTEC are characterized as Epcam^{+}, CD205^{+}, β5t^{+}, keratin 8^{+}, CD40^{+} or MHCII^{+} cells. cTEC express cytokines such as DLL4 and IL-7, which promote the differentiation of T-cell progenitors, thereby inducing initial T-cell development [377]. mTEC are characterized by the expression of MHC^{high}, CD40^{+}, UEA^{+}, CD80^{high}, keratin 5/14 and TSA controlled by autoimmune regulator (AIRE) [378]. Tissue specific antigens (TSA) expression by mTEC confers the capacity to directly present self-antigens to achieve negative selection of T-cells [378]. This characteristic allows mTEC to present peripheral self-antigens to developing T-cells. Moreover, mTEC also produce thymus stromal lymphopoietin (TSLP) and ICOS-L that promote differentiation of thymic regulatory T-cells (CD4^{+}CD25^{+}FOXP3^{+}) [379, 380]. Therefore, mTEC play a critical role in the establishment of central immune tolerance.

mTEC frame a classical structure found in the human thymus, the Hassall's corpuscles. These structures are considered as the burial ground of dead T-cells [381]. Nevertheless, Hassall's corpuscles are also active structures that express cytokines such as TGF-α, SDF-1, IL-7 and TSLP, a critical cytokine that activates DC to upregulate MHC-II [382].

Thymic DC are bone-marrow derived cells that represent around 0.5% of the cellular components in the thymus [383]. There are three subpopulations of DC in the thymus. Resident dendritic cells represent more than half of all DC in the thymus. This subpopulation localize in the medulla and are characterized as CD8α^{+}SIPRα^{-} [384]. They participate in the negative selection process though the presentation of self-peptides transferred from mTEC and in the differentiation of thymic Treg, as they express the co-stimulatory molecules CD80 and CD86 required for CD28 signaling in Treg [379]. The two other subpopulations of DC are migratory DC and plasmacytoid DC. These subpopulations are present in the corticomedullar zone of the thymus and are specialized in the presentation of peripheral antigens. Migratory and plasmacytoid DC are characterized as CD8α^{-}CD11b+SIPRα^{+} and CD11^{int} CD45RA^{int}, respectively [384, 385].

2.5.1.1.3. Thymic myoid cells

Myoid cells are a rare cell population found in all vertebrates [386]. Thymic myoid cells are located in the medulla and the corticomedullar junction [350]. Their main feature is the expression of

muscle-specific proteins such as desmin, troponin T, the myogenic transcription factor MyoD1 and acetylcholine receptor [387, 388]. Thymic myoid cells play an important role in the thymus, as they protect thymocytes from apoptosis, through the activation of ERK1/2 and Akt pathways [389]. Contrary to TEC, thymic myoid cells do not express MHC and therefore do not present antigens. However, they can be a source of autoantigens to DC [390].

2.5.1.1.4. Macrophages

During T-cell development, around 95% of thymocytes are eliminated by apoptosis after positive and negative selection and must be removed [353]. Thymic macrophages are stromal cells found in both cortical and medullar zones and are identified as $CD68^+$, $ED1^+$ and F4/80 [391]. Their thymic main role is the phagocytosis of apoptotic cells [392].

2.5.1.2. Pathological $AChR^+$ MG thymus

2.5.1.2.1. Thymoma

Thymoma is a thymic tumor caused by an abnormal development of epithelial cells. The presence of a thymoma is linked to the development of AIDs such as SLE, erythroblastopenia and, most commonly, MG [269, 393, 394]. MG-associated thymoma is a benign to low-grade malignant tumor characterized by an abnormal development of thymic cortical epithelium [395, 396]. According to the World Health Organization, thymomas are classified as type A, AB, B1, B2 or B3 based on the characteristics of epithelial cells and the presence of immature thymocytes [393]. Thymoma is present in around 10–20% of patients with generalized MG [269]. MG patients commonly present lymphocyte-rich tumors, classified as thymoma B1 or B2 [396]. The high incidence of autoimmune diseases related to thymoma has been linked to defective positive and negative selection that leads to leakage of autoreactive T-cells [397]. Interestingly, studies have shown that most thymomas harbor a decrease in AIRE expression, the transcription factor regulating the central tolerance process. However, no direct link has been established for MG development [398]. Finally, patients with thymoma develop anti-IFN-α antibodies as well as antibodies targeting IL-12[399].

2.5.1.2.2. Lymphofollicular thymus

Thymic lymphoid hyperplasia is the classical characteristic of EOMG AChR$^+$ MG patients. 65% of MG patients display thymic follicular hyperplasia. Hyperplastic MG thymus can be considered as a tertiary lymphoid organ (TLO). TLOs are characterized by an infiltration of B-cells, development of a vascular system, presence of high endothelial venules (HEV), presence of APC and overexpression of pro-inflammatory cytokines and chemokines [400, 401]. TLOs are normally triggered by immunization or infection. However, in AIDs, these structures are usually due to chronic inflammation [400].

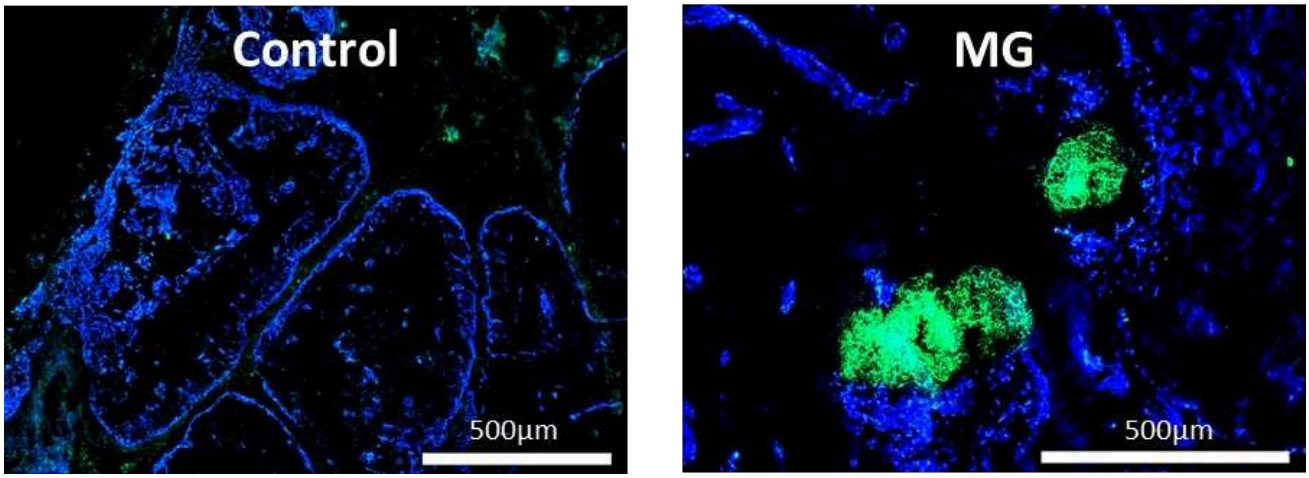

Figure 11 : Hyperplastic MG thymus with infiltration of B-cells
AChR$^+$ MG thymus is characterized by the abnormal infiltration of B-cells, which organize in ectopical germinal centers where they become antibody-producing B-cells. Keratin 5/14 in blue, CD20 in green

The infiltrated lymphoid cells organize in eGCs in the medullar zone of the thymus [395, 402] (**Figure 11**). Indeed, MG mTECs present an overexpression of the chemokine CXCL13, which attracts B-cells through CXCR5 [403]. Hyperplastic MG thymuses display an increased number of HEV and an overexpression of VEGFR3, a lymphatic endothelial cells (LEC) marker [404]. Both HEV and LEC are associated with lymphocytes homing into secondary lymphoid organs. Moreover, HEV and LEC in a hyperplastic MG thymus present an overexpression of CXCL12 and CCL21, respectively [403, 404].

Of note, **Figure 12** summarizes the GC development and the different cells' reactions occurring in the eGCs in the inflamed tissue [405]. Formation of GCs start when a naïve B-cell meets an antigen and migrates to the T-cell zone, where it receives co-stimulatory signals from CD4$^+$ T-cells to

proliferate. Then highly proliferative B-cells, or "centroblasts," migrate to the dark zone (DZ) attracted by CXCL12 as they express the receptor CXCR4. While in the DZ, B-cells undergo a process called somatic hypermutation. This process is controlled by the expression of activation-induced deaminase (AID). AID induces point mutations in the variable region of the B-cell receptor (BCR) and therefore generates immunoglobulins with different affinities [406]. B-cells then migrate to the light zone (LZ) following a CXCL13 gradient produced by follicular dendritic cells (FDC). Within the LZ, B-cells, or "centrocytes," interact with antigens trapped in the surface of FDC [407]. B-cells next present antigens to T follicular helper (Tfh) cells also found in the LZ. Tfh cells are critical in this process, as they are responsible for the positive selection of B-cells with higher affinity BCR [406]. Selected B-cells can either re-enter into the DZ to undergo somatic hypermutation once again or exit the GC and mature as long-lived plasma cells that produce antibodies or memory B-cells [407, 408]**.** Thus, germinal centers are highly dynamic structures with a crucial role in control of the immune response and autoimmunity.

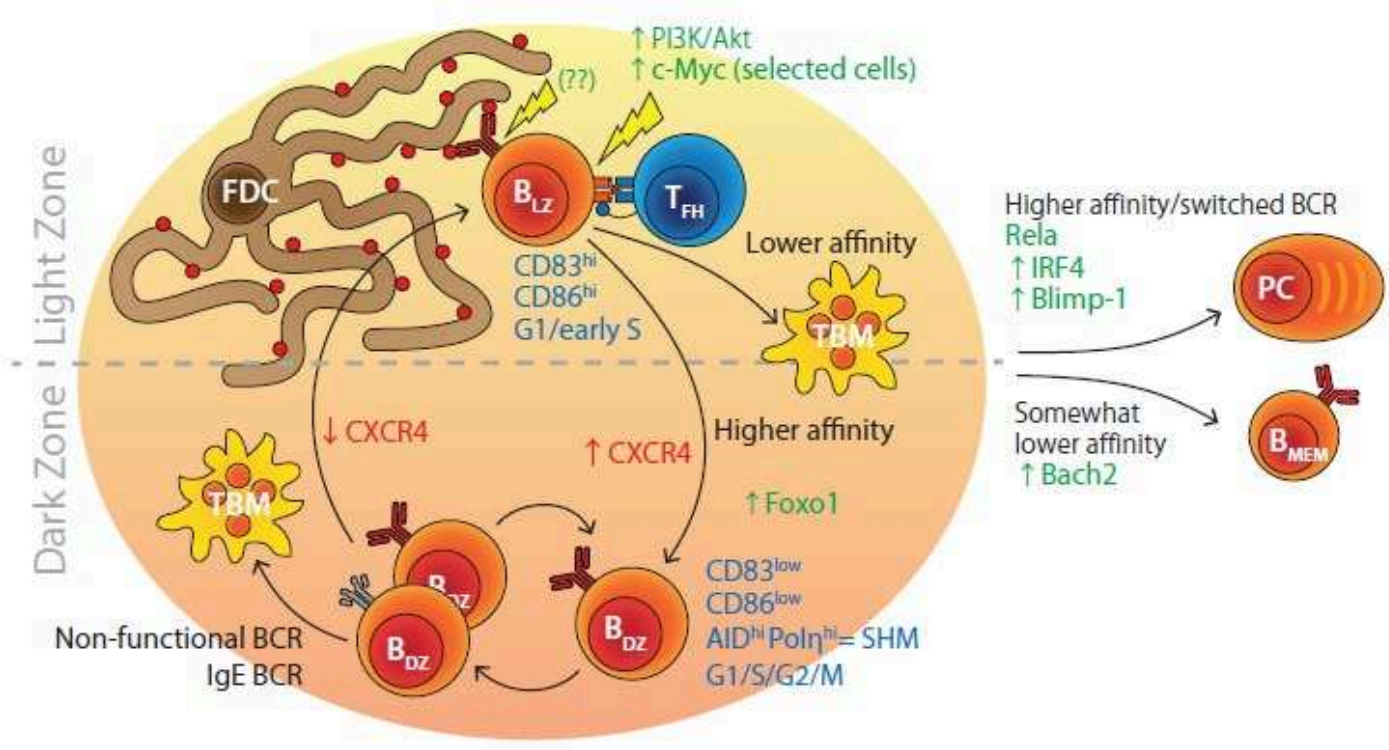

Figure 12 : Organization and cellular components of germinal centers

Germinal centers are structures where T- and B-cells interact to induce the differentiation of B-cells into plasma cells. B-cells that received the stimulation signal by T-cells migrate to the dark zone to undergo somatic hypermutation of their BCR. After this process, B-cells migrate back to the light zone to interact with antigens presented by follicular dendritic cells and to present antigens to T follicular helper cells. Tfh cells positively select B-cells with high-affinity BCR to become antibody-producing cells. [405].

The tight relation between thymic eGCs and MG has long been known [409]. Perlo et al. have shown that the improvement of the clinical symptoms of MG patients who underwent thymectomy is correlated to the degree of thymic hyperplasia [409]. Moreover, the concentration of circulating antibodies is directly correlated with the degree of hyperplasia, which can be significantly reduced with glucocorticoid therapy [284]. A clinical trial has confirmed that thymectomy ameliorates clinical symptoms in MG patients and allows a dose reduction of corticoids [410].

As a result of the presence of eGCs, hyperplastic MG thymuses exhibit diverse modifications. **1)** A high number of B-cells overexpress Bcl-2 (30% versus 1–3% in control thymuses). Of note, overexpression of Bcl-2 indicates enhanced survival [411]. **2)** Expression of activation-induced cytidine deaminase is increased, reflecting that B-cells found in eGCs undergo somatic hypermutation [412]. **3)** An increased number of Tfh cells participate in B-cell selection [413].

2.5.1.2.3. Treg in AChR$^+$ MG thymus

The MG thymus presents a disequilibrium between Treg and Th17 cells. While the number of thymic Treg cells from AChR$^+$ MG patients does not differ from normal thymuses, their suppressive activity is impaired, and their expression of FoxP3 is decreased [414-416]. FoxP3 expression in Treg cells is in part controlled by the phosphorylation of signal transducer and activator of transcription 5 (STAT5) induced by IL-2 signaling. In ACHR$^+$ MG thymuses, IL-2 is overproduced [417], therefore the low level of FoxP3 in Treg cells is most likely due to a decreased phosphorylation of STAT5 after IL-2 signaling [338]. In addition, thymic MG Treg cells are plastic and show an overexpression of pro-inflammatory cytokines including IL-17 and IL-21, both classically expressed by Th17 cells [417]. This suggests that Treg cells can acquire a Th17-like phenotype upon exposure to the inflammatory milieu. Additionally, cytokines such as IL-6, IL-1β and TGF-β1 are overexpressed in the MG thymus and create the perfect environment for the development of inflammatory T-cells [93, 418, 419].

2.5.1.2.4. Th17 cells in AChR$^+$ MG thymus

I have written a review compiling the studies and the state of the art on the status of Th17 balance in AChR$^+$ MG thymuses [420]. This review has been included at page159 in this thesis manuscript. Briefly, different groups have provided evidence of Th17 cell involvement in MG thymuses [271, 417, 421, 422]. Moreover, increased levels of IL-17 in the serum [421] and plasma [422] of MG patients are associated with MG severity, suggesting a role of this molecule in the pathogenic mechanisms occurring in MG. In addition, it has been shown that Th17 cells, in other autoimmune

diseases, may also contribute to maintain eGC structures and then promote differentiation of B-cells and the production of antibodies [201, 423].

2.5.1.2.5. Deregulation of IFN-I in ACHR^{+} MG thymus

The AChR^{+} MG thymus is characterized by an overexpression of type I interferon (IFN-I) due to an activation of TLRs. Type I interferon (IFN-I) is a family of cytokines that are known for their role in innate immunity and can be produced by different cell types after infection with pathogen such as viruses. The MG thymus displays an Epstein-Barr virus (EBV) infection signature that correlates with an overexpression of TLR7 and TLR9 [327]. TLR9 and TLR7 are intracellular endosomal lysosomal receptors that recognize bacterial and viral DNA respectively. This observation suggests that activation of the IFN transduction pathway in MG thymus is due to viral infections.

Cufi et al. showed that activation of TLR3 with poly(I:C) (a molecule that mimics a double-strand RNA virus) induces molecular changes in mTEC. Indeed, poly(I:C) induced an overexpression of the α-AChR, increasing expression of CXCL13, CCL21 and BAFF through IFN-I signaling [324]. Of note, IFN-I regulates mTEC expression of α-AChR only and no other TSA while increasing mTEC death [311]. Therefore, it has been hypothesized that AChR fragments could be captured by IFN-I-activated DC and trigger autosensitization within the thymus [311, 424].

There have been efforts to model in vivo the effects of IFN-I activation in the thymus. For instance, Robinet et al. showed that, in EAMG, poly(I:C) potentializes myasthenic clinical signs in mice and stimulates a higher concentration of anti-torpedo acetylcholine receptor (anti-TAChR) antibodies [425]. These observations could be a consequence of an overexpression of IFN-β, CXCL13, CCL21, and α-AChR induced by poly(I:C). Robinet et al. also showed a recruitment of B-cells, but without GC formation [425]. Therefore, more must be done to discover the implications and consequences of the deregulation of IFN-I in thymic MG physiological changes.

2.5.2. The muscle

2.5.2.1. Neuromuscular junction physiology

Skeletal muscle derives from the mesodermal cells that are committed to a myogenic phenotype. After their division, they differentiate into myoblasts that align and fuse to form myotubes [426]. Acetylcholine receptor initial expression starts at the myoblast stage and is upregulated in the

myoblast fusion process to form myotubes. Clustering of AChR in the muscle is regulated by the production of agrin by the motor neuron. Indeed, the formation of the NMJ requires a crosstalk between the nerve and the muscle. Motor axons that reach the muscle produce agrin, which binds to the LRP4 receptor and activates the phosphorylation of MuSK [427]. Motor axons produce acetylcholine (ACh), which activates AChR.

The NMJ or motor endplate is the site of transduction of electrical signals from the neuron into a chemical signal that induces contraction of the muscle. Acetylcholine is synthetized in the terminal axon from acetyl CoA and choline by the action of the choline acetyl transferase. This neurotransmitter is stocked into vesicles that, after electrical stimulation of the nerve, fuse with the presynaptic membrane to release their acetylcholine content into the synaptic cleft. This signal induces the clustering of AChR at specific sites in the myotubes to form the NMJ [426, 427] (**Figure 13**). In the postsynaptic membrane, AChRs are found at the crest of the junctional folds [426]. AChRs are composed by five different subunits—α1, β1, γ, ε and δ—that arrange in a tubular order.

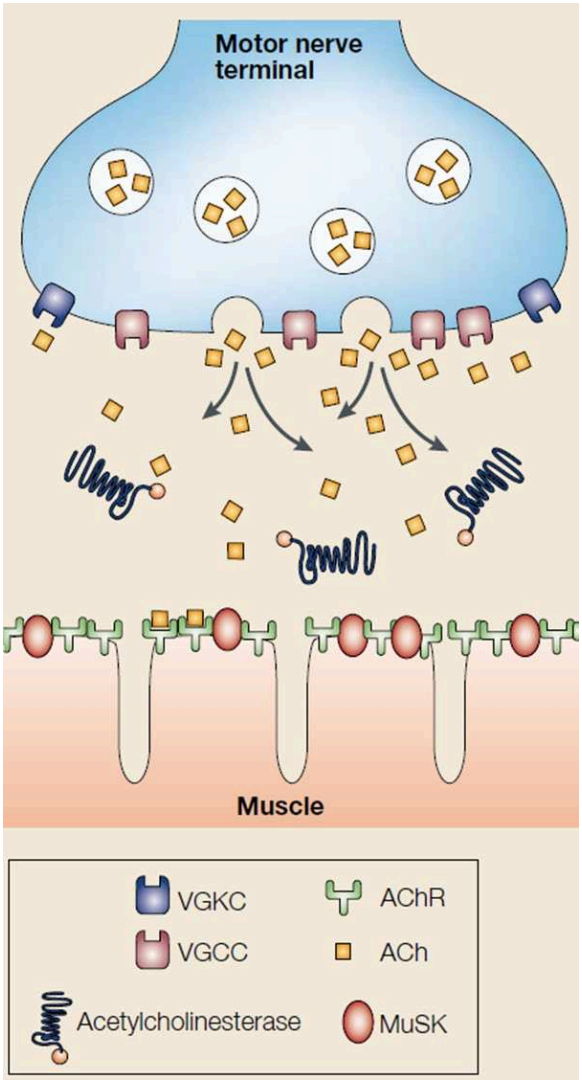

Figure 13 : Neuromuscular junction in physiological conditions
The neuromuscular junction is the site of transduction of electric signals from the CNS to chemical signals and muscle contraction. The motor nerve terminal contains synaptic vesicles with acetylcholine (ACh) that, after electrical impulse, fuse with the terminal membrane and release the contained ACh to the synaptic cleft. In the zone, acetylcholinesterase limits the time of action of ACh. The clustered acetylcholine receptors (AChR) are located in the postsynaptic membranes. AChR are activated by ACh to act as ion channels and induce the depolarization of the muscle and muscle contraction [256].

According to its composition there are two types of AChRs. **1)** Fetal AChR is composed of two alpha subunits and one subunit each of β, γ and δ. Fetal AChR is required for the formation of the neuromuscular junction [428]. **2)** Adult AChR is composed of two subunits of α and one subunit each of β, ε and δ. There are two types of AChRs: muscarinic AChR (mAChR) and nicotinic AChR (nAChR). mAChR are mostly present in the CNS and are part of the G-protein-coupled receptor family, while nAChR are present in the CNS and the NMJ. The nAChR is a ligand-gated ion channel. Activation of nAChR by acetylcholine induces conformational changes that open the channel to allow Ca^{2+} influx, depolarization of the muscle fiber and the consequent contraction of the muscle [429, 430]**.**

2.5.2.2. Skeletal muscle regeneration

The adult skeletal muscle is a complex and heterogeneous tissue with the ability to adapt and regenerate due to stimulus such as exercise, growth or damage. The capacity of adult muscle to regenerate relies on muscle stem cells also known as satellite cells (SC). Satellite cells are myogenic precursors normally found in a quiescent state between the sarcolemma and the basal lamina of myofibers [431]. Satellite cells are characterized by the expression of the paired box transcription factors 3 and 7 (Pax3 and Pax7). These transcription factors control the development of myogenic cells [432]. After activation, SC enter the cell cycle and start to express the transcription factor MyoD (a marker of myogenic commitment) to differentiate into myoblasts. Then, myoblasts downregulate Pax7 and upregulate MyoD and Ki67. Myoblasts differentiate into myocytes that express Myogenin (MyoG) and fuse with each other or with existing fibers to generate myotubes [433, 434] (**Figure 14**)**.**

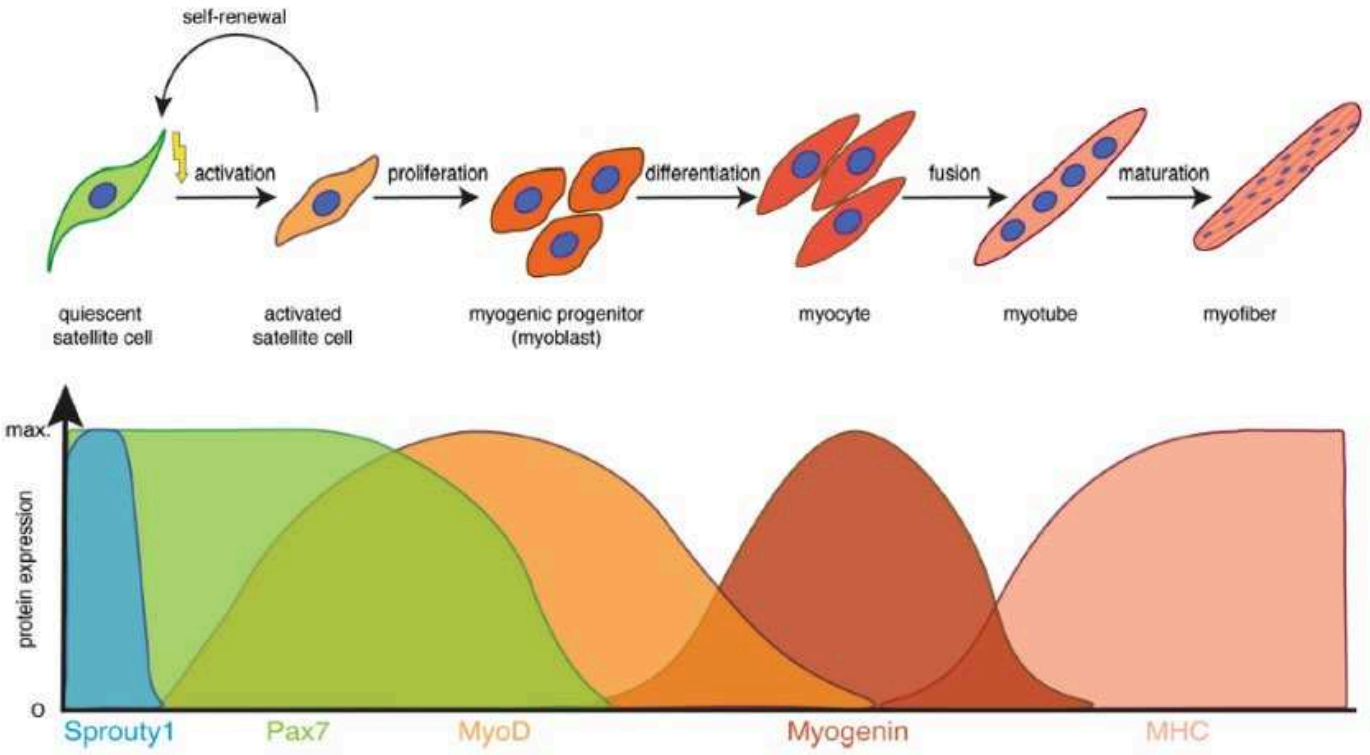

Figure 14 : Regeneration of muscle fibers

Muscle regeneration is a sequential process that can be followed by the expression of different transcription factors. Activation of Pax7⁺ satellite cells induces their proliferation and the co-expression of the transcription factor Pax7 and MyoD. At this point, a portion of satellite cells return to a quiescent state, downregulate MyoD and upregulate Sprouty1. The rest continue their differentiation program and downregulate Pax7⁺ to emerge into a myogenic progenitor known as a myoblast. Committed myoblasts then proliferate by the expression of Ki67⁺ to acquire a myocyte phenotype that downregulates MyoD and upregulates Myogenin (MyoG). Then multinucleated myotubes formed by the fusion of myocytes downregulate MyoG. Finally, myotubes delocalize nuclei to the periphery and express myosin heavy chain to acquire the myofiber phenotype. Image from [433].

2.5.2.3. Effects of myasthenia gravis on muscle physiology

Although the main clinical symptom of MG patients is muscular fatigue, the consequences in the physiology of the NMJ and the muscle of the presence of autoantibodies are not well known.

As shown in **Figure 15**, there are three mechanisms by which anti-AChR antibodies act on the NMJ: the NMJ degradation complement activation mediated, the AChR internalization and the AChR blocking. These processes promote the loss of AChR at the NMJ [276-279, 435, 436]. However, studies have shown that, compared with normal muscle, MG muscle attacked by autoantibodies

presents an increased mRNA expression of AChR. This is considered to be a compensation mechanism of MG muscle to overcome autoantibody attacks [437].

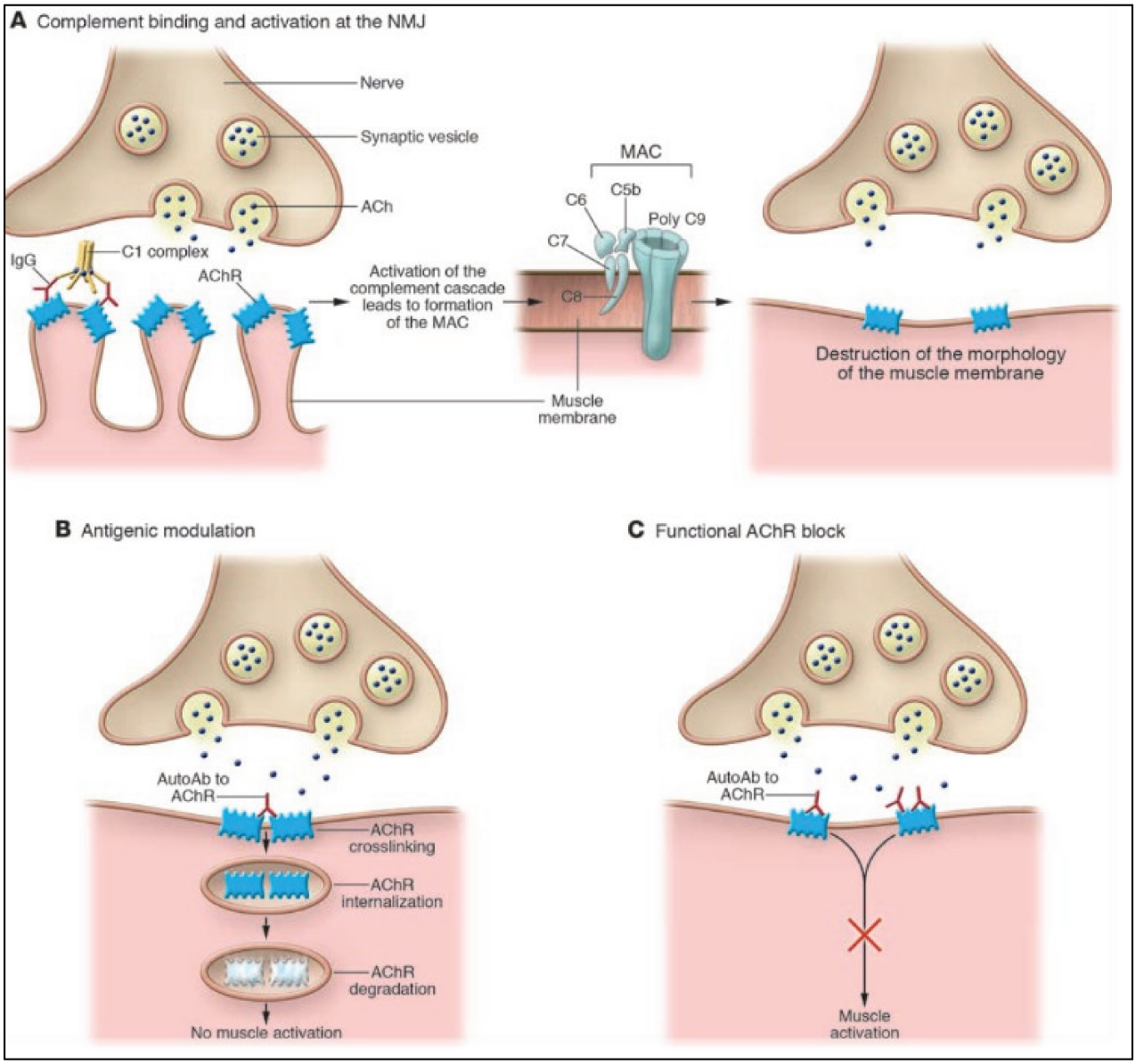

Figure 15 : *Effects of anti-AChR antibodies in the neuromuscular junction*

Myasthenia gravis' hallmark is the attack of AChR present in the NMJ by autoantibodies. Anti-AChR antibodies can act by three different mechanisms. A) Activation of the complement pathway and the formation of a membrane attack complex that produces structural changes in the postsynaptic NMJ. B) Antibodies also have the capacity to crosslink AChRs, which induces the internalization and degradation of the receptors. C) Anti-AChR antibodies can act as blocking antibodies, reducing the binding of acetylcholine to its receptor. from [438].

Attack by anti-AChR antibodies induces changes in MG muscle metabolism. Maurer et al. showed that the presence of anti-AChR antibodies induces an overexpression of IL-6 and IL-6R in muscle of EAMG rats and in human biopsies [439]. Analysis in vitro showed that Akt, a downstream target of IL-6 and insulin like growth factor (IGF-1) involved in growth and glucose intake, was also affected by anti-AChR antibodies [439]. Furthermore, anti-AChR antibodies activate muscle SC. Recently, our team showed that human MG muscle harbors an increased number of activated SC ($Pax7^+$ $MyoD^+$ $Ki67^+$) and an altered myoblast differentiation and fusion induced by the presence of autoantibodies [440]. These studies show that the attack of muscle by anti-AChR antibodies has a broad impact not only at the NMJ but also in the cell's metabolic pathways as well as in the SC ability to regenerate.

2.6. $AChR^+$ myasthenia gravis animal models

Myasthenia gravis animal models have been used for more than 30 years. The first MG animal model was induced by passive transfer of antibodies from MG patients' sera in mice and rabbits. Different types of MG animal models have been developed based on three main methods: active induction, passive induction or adoptive transfer.

2.6.1. Active induction of myasthenia gravis

The active induction of AChR myasthenia gravis mouse model is the experimental autoimmune myasthenia gravis (EAMG). The first report of this model showed that purified acetylcholine receptor obtained from eel electric organ induced myasthenic symptoms in rabbits [263]. Today, this model remains the most used model to study myasthenia gravis. EAMG model is based in the injection of AChR epitopes to brake immunological tolerance and induce the production of antibodies against the exogenous and self AChR. Thus, this model mimics the MG muscle symptoms EAMG model is widely used in rats (Lewis rat) and mice (C57Bl/6, SJL, and AKR mouse strain). Of note, the rate of myasthenic mice after immunization is around 50–70% [441].

The myasthenic symptoms observed in this model mimic some of the human MG symptoms, as animals present anti-AChR antibodies, deposition of antibodies at the NMJ and complement activation, loss of muscle AChR and activation of T- and B-cells. The possibility of inducing EAMG in mutant mice makes this model ideal for understanding the mechanisms behind MG. However, EAMG's main limitation is the exclusion of the thymus as the effector tissue. Therefore, this model does not allow us to understand the pathogenesis related to thymic changes [442].

Of note, active immunization is also used to induce MG associated with the presence of anti-MuSK or anti-LRP4 antibodies [443, 444]. In both models, immunization induces the presence of specific autoantibodies against MuSK and LRP4 and causes decrease of muscular strength and the loss of NMJ organization [443, 444].

2.6.2. Passive induction of myasthenia gravis

Myasthenia gravis can also be reproduced in animals by passive induction. This model relies on the continuous injection of purified anti-AChR antibodies from either EAMG-immunized mice or MG patients [445]. The strength of this model is the rapid development of myasthenic symptoms (i.e., muscle weakness). Thus, this model is useful to determine mechanisms of action of anti-AChR antibodies at the NMJ as well as possible therapies that are directed to control the pathogenicity of antibodies, such as complement inhibitors or antibody inhibitors. However, passive transfer of anti-AChR does not aim to disturb the immune system, therefore this model has limited applications [442, 445].

2.6.3. Adoptive transfer of myasthenia gravis.

Human $AChR^{+}$ myasthenia gravis originates in the thymus. Adoptive transfer of MG is based in the transplantation of thymic cells or tissue from MG patients into severe combined immunodeficiency mice (NOD-SCID gamma C KO or NSG). NSG mice are immunodeficient, without a functional immune system due to an impairment in the rearrangement of B- and T-cell receptors and the lack of expression of IL-2Rγ [446, 447]. This mouse strain accepts the engraftment of exogenous tissue without an immunological reaction.

The NSG mouse model has demonstrated that the thymus contains all the components required to induce MG. The first report of adoptive transfer MG showed that thymus engrafted beneath the renal capsule was able to release anti-AChR antibodies into the mouse's circulatory system [448]. Moreover, this report also showed that human anti-AChR antibodies are able to bind to murine AChR. Intriguingly, there were no circulating human cells, and mice do not show clinical symptoms of MG [448]. We have developed an NSG-MG mouse model by subcutaneous engraftment of MG thymus into SCID mouse. This model shows many similarities to human MG, such as the presence of anti-AChR antibodies, circulating B- and T-cells and muscular weakness. Interestingly, recipient mice mimicked the clinical symptoms observed in human thymic donors. Moreover, the engrafted

thymus presented functional structures such as cortical and medullar zones and the presence of T- and B-cells up to 8 weeks after transplantation [449]. Thus, this model opens the possibility to directly study human MG thymus and the pathogenesis involved while determining the clinical impacts of possible therapies.

3. MG therapies

3.1. Decision tree for treatment of MG

According to the international consensus guidance for MG management [450], the treatment algorithm for MG patients is shown in **Figure 16** [451].

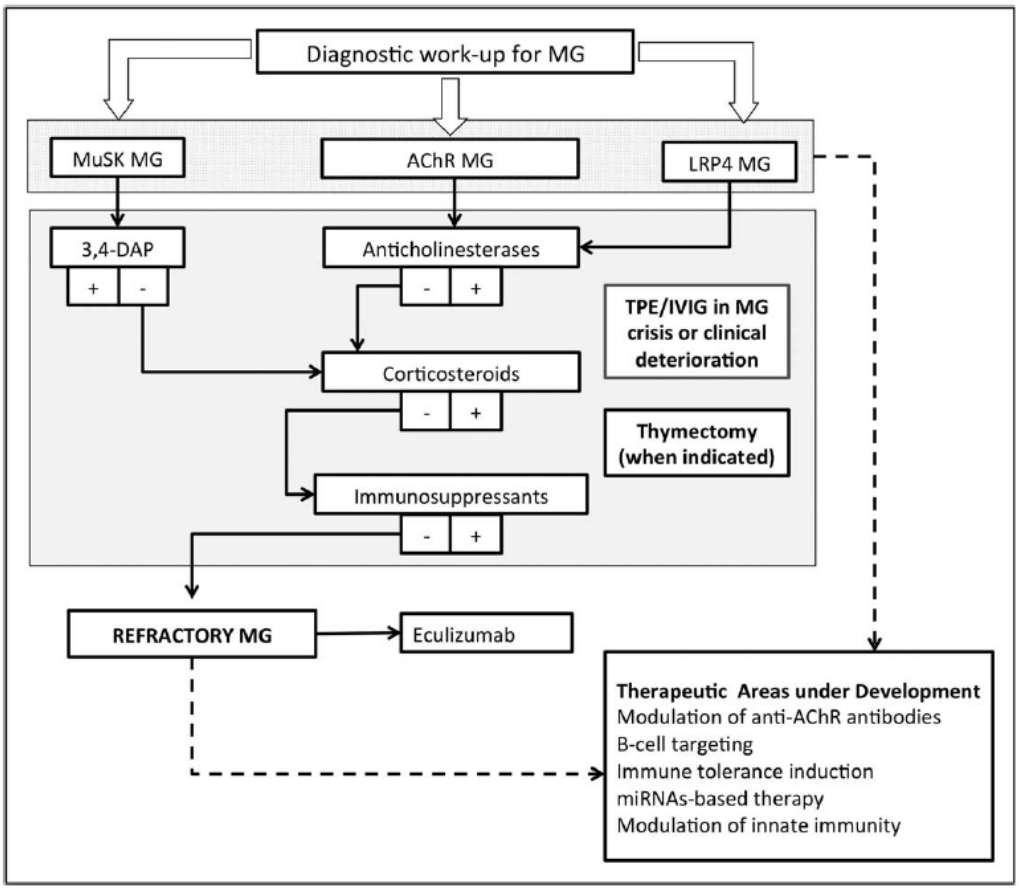

Figure 16 : Algorithm for the treatment of MG

Treatment of myasthenia gravis main forms (MuSK, AChR & LRP4). Anticholinesterases are the first-line treatment for LRP4 and AChR$^+$ MG, while MuSK first-line treatment is with 3,4 diamino pyridine. Corticosteroids followed by immunosuppressants are common treatments in these types of MG. Plasma exchange and IVIg are also common treatments when patients present myasthenic crisis. Thymectomy is indicated for AChR$^+$ patients with thymic hyperplasia or thymoma. Biological treatment with eculizumab is indicated in the three subtypes when MG is refractory. from [451].

3.1.1. Inhibitors of Acetylcholinesterase

The anticholinesterase drugs increase the availability of ACh by inhibiting the biological activity of acetylcholinesterase at the NMJ. It is the first-line medication after the diagnosis of $AChR^+$ MG. This treatment is only symptomatic, with a good response in most patients. The most common drug is pyridostigmine bromide [303]. Long-term use of pyridostigmine is useful for patients with very mild symptoms or with no progressive MG. However, since this drug does not prevent progression of the pathology, most patients eventually require therapy changes [452].

3.1.2. Corticosteroids

There are two types of corticosteroid: mineralocorticoids and glucocorticoids. Mineralocorticoids regulate sodium and water levels, while glucocorticoids regulate metabolism and immune activation. Glucocorticoids are the second line of treatment for MG patients. The effects of glucocorticoids are mediated by the cytosolic glucocorticoid receptor, which after activation translocate to the nucleus and binds to the DNA-binding site named glucocorticoid response element to induce the expression of IL-10 and annexin A1. The complex glucocorticoid-glucocorticoid receptor also interacts with NF-κB. This interaction prevents NF-κB DNA-binding and synthesis reduction of pro-inflammatory proteins [453, 454].

The most common drugs of this group are prednisone and prednisolone [452]. The beneficial effect on MG patients treated with glucocorticoids has been linked to a decreased number of germinal centers present in the thymus [403]. However, long-term therapy with glucocorticoids has many undesirable side effects, including the presence of acne, changes in mood, hypertension, hyperglycemia and a predisposition to infections [455]. Thus, administration of glucocorticoids must be tightly controlled by the physician to avoid side effects.

3.1.3. Immunosuppressors

Immunosuppressors are drugs that control the proliferation of T- and B-cells. Their mechanisms of actions include the blockade of the cell cycle and inhibition of purine synthesis. The most common immunosuppressors are azathioprine, mycophenolate mofetil, cyclosporine, tacrolimus, cyclophosphamide and methotrexate [456]. Immunosuppressant therapy has a delay of action between 1 and 6 months. Therefore, this therapy is usually given as a steroid-sparing therapy until improvement of symptoms allows an eventual reduction in glucocorticoid doses.

Immunosuppressors are a long-term therapy and the therapy most used for the treatment of MG, with a response rate around 75% [457]. However, even if this group of drugs has a good rate of response, they present a challenge in avoiding the inherent side effects, as shown in **Table 1.**

Drug	Mechanism of action	Adverse effects (listed in order of frequency)
Azathioprine	• Purine analogue • Interferes with DNA synthesis • Reduces T cell and B cell proliferation	Hepatic enzyme increase, nausea, macrocytosis, leukopenia, thrombocytopenia, pancreatitis, hair loss and idiosyncratic reaction
Mycophenolate mofetil	• Prodrug • Inhibits de novo purine synthesis • Reduces T cell and B cell proliferation	Nausea, diarrhoea, leukopenia, liver enzyme increase and hypertension
Cyclosporine	• Calcineurin inhibitor • Reduces IL-2 transcription • Blocks T cell activation	Nephrotoxicity, hypertension, tremor, hypertrichosis, gingival hyperplasia, dizziness, headache and encephalopathy
Tacrolimus	• Calcineurin inhibitor • Reduces IL-2 transcription • Blocks T cell activation	Hypertension, tremor, diabetes mellitus, nephrotoxicity, myocardial hypertrophy and encephalopathy
Methotrexate	• Folate analogue • Interferes with DNA synthesis • Reduces T cell and B cell proliferation	Stomatitis, nausea, hair loss, leukopenia, macrocytic anaemia, thrombocytopenia, oligospermia and hepatic enzyme increase
Cyclophosphamide	• Alkylating agent • Interferes with DNA replication • Suppresses B cells more than T cells	Nausea, vomiting, fever, hair loss, liver dysfunction, liver enzyme increase, cystitis, oligospermia and myelosuppression

Table 1 : Immunosuppressors used in treatment of MG

Immunosuppressors are the most common long-term treatments in myasthenia gravis. While they have a good rate of response in patients, immunosuppressive drugs come with a variety of adverse effects that must be considered by the physician. Adapted from [452].

3.1.4. Intravenous immunoglobulins and plasmapheresis

Intravenous immunoglobulins and plasmapheresis are fast-acting and short-term therapies used in acute severe cases of MG. IVIg acts by inhibition of complement deposition, modulation of inflammatory cytokines and blockade of fragment crystallizable receptor (Fc receptors) [456].

Plasmapheresis is based in the removal of the pathogenic factors in circulation such as antibodies, complement and cytokines. IVIg and plasmapheresis are proven to be efficient in controlling myasthenic crisis but not as long-term treatment in chronic patients [456, 457]. IVIg is normally well

tolerated except for rare cases of MG patients with IgA deficiency. The main restriction of plasmapheresis is the requirement for a specialized hospital setting and good central venous access to avoid catheter problems and infections.

3.1.5. Biologics treatments

Biologic treatments are emerging therapies that aim to specifically target a component of the immune system. The accumulating knowledge of the physiopathological mechanisms allows us to determine the critical factor that can induce an amelioration. Rituximab and eculizumab are the only approved biological therapies to treat MG. These biological treatments are indicated in refractory MG patients [458]. Rituximab is a monoclonal antibody that targets CD20 on B-cells. It induces complement-dependent cytolysis and cell-mediated cytotoxicity, thus depletion of mature B-cells. Eculizumab is a monoclonal antibody that targets the C5 complement component and blocks the formation of the membrane attack complex in the muscle [456, 459]. Clinical trials are still in progress or have been recently completed. So far, Rituximab has shown a good efficacy on $MuSK^+$ patients, with around 70% of patients showing an improvement while only 30% of $AChR^+$ MG patients showed improvement [460]. The efficacy of eculizumab remains to be determined, as clinical trials have shown only a slight amelioration in MG patients. Since eculizumab does not target all the immunopathological components of MG (i.e., T- and B-cells), patients still required immunosuppression therapy [452].

3.1.6. Thymectomy

Thymectomy has been used as a therapy for MG since the beginning of 20^{th} century. However, it was only in recent years that the effectiveness of this procedure was evaluated. Wolfe and colleagues carried out a clinical trial to evaluate the benefits of thymectomy on patients with $AChR^+$ generalized MG. The results of this study showed that thymectomy improves the clinical symptoms for up to 5 years, allows reduction of glucocorticoid dosages and induces limited requirements for immunosuppressor therapy [410, 461]. To date, thymectomy is indicated in recently diagnosed EOMG patients and in patients with thymoma. Of note, thymectomy is not a cure, and patients still require long-term therapy.

3.2. Clinical trials ongoing

Clinical trials on MG are challenging due to the diversity and subtypes of MG and the small number of patients. However, there are efforts to carry out clinical trials to determine the efficiency of classic therapies and/or to test emerging therapies.

As mentioned above, immunosuppressive drugs and thymectomy are common therapies for MG patients. Recently, results from clinical trials of these therapies have been published with interesting results [462] [410]. A clinical trial carried out in the U.S. showed that methotrexate (an immunosuppressive drug) has no beneficial effect as a steroid-sparing drug in $AChR^{+}$ MG patients over 12 months [462]. These results show that even if MG can be controlled, therapy is life-lasting, and a need for less invasive treatments and more specific targets remains. In this line, biological therapies that use specific monoclonal antibodies are emerging and are being tested in MG.

As of June 2019, there are 82 registered clinical trials linked to myasthenia gravis.

CLINICAL TRIAL NUMBER	STATUS	PHASE	CONDITION	INTERVENTION	THERAPEUTIC APPROACH
NCT03669588	Recruiting	3	Generalized Myasthenia Gravis	Biological: ARGX-113	Neonatal Fc Receptor blocker
NCT03770403	Recruiting	3	Generalized Myasthenia Gravis	Biological: ARGX-113	
NCT03772587	Recruiting	2	Generalized Myasthenia Gravis	Drug: M281	
NCT03863080	Not yet recruiting	2	Myasthenia Gravis	Drug: RVT-1401	
NCT03896295	Not yet recruiting	2	Generalized Myasthenia Gravis	Drug: M281	
NCT02301624	Active, not recruiting	3	Refractory Generalized Myasthenia Gravis	Biological: Eculizumab	Anti-C5 monoclonal antibody
NCT03759366	Recruiting	3	Myasthenia Gravis Myasthenia Gravis, Juvenile Myasthenia Gravis, Generalized	Drug: Eculizumab	
NCT03920293	Recruiting	3	Generalized Myasthenia Gravis	Biological: Ravulizumab	
NCT01727193	Recruiting	3	Myasthenia Gravis	Drug: Azathioprine Drug: Leflunomide	Immunosuppressive drug
NCT03490539	Recruiting	Observational	Neurological Disorder Autoimmune Diseases	Drug: Mycophenolate Mofetil Drug: Azathioprine	
NCT02774239	Recruiting	3	Myasthenia Gravis	Drug: Human normal immunoglobulin G (IgG)	Subcutaneous Immunoglobulin
NCT02100969	Active, not recruiting	2	Myasthenia Gravis	Drug: HIZENTRA®	

CLINICAL TRIAL NUMBER	STATUS	PHASE	CONDITION	INTERVENTION	THERAPEUTIC APPROACH
NCT03304054	Recruiting	3	Myasthenia Gravis, Generalized	Drug: Amifampridine Phosphate	Nonspecific blocker of potassium channel
NCT03579966	Recruiting	3	Myasthenia Gravis, MuSK	Drug: Amifampridine Phosphate	
NCT02950155	Recruiting	3	Generalized Myasthenia Gravis	Drug: Rituximab	Anti-CD20 monoclonal antibody
NCT03510546	Recruiting	4	Myasthenia Gravis	Drug: Pyridostigmine	Acetylcholinesterase
NCT02102594	Recruiting	2	Myasthenia Gravis Systemic Lupus Erythematosus Rheumatoid Arthritis	Drug: Bortezomib	Proteosome inhibitor
NCT03315130	Active, not recruiting	2	Generalized Myasthenia Gravis	Drug: RA101495	Synthetic peptide blocker of C5
NCT03914638	Recruiting	2	Myasthenia Gravis	Drug: Salbutamol 4mg Tablet	β2 agonist
NCT03059888	Recruiting	Early phase 1	Myasthenia Gravis	Drug: Abatacept Injection	Regulator of T-cell activation

Table 2 : Current clinical trials for the treatment of myasthenia gravis
Clinical trials registered in www.Clinicaltrials.gov

Among the registered clinical trials, five of them aim to determine the effectiveness of a therapy that targets the neonatal Fc receptor (FcRn). This therapy is based on blocking the natural process of recycling IgG. Normally, IgGs are recycled by endothelial cells through a process called pinocytosis that uses the Fc receptor (FcR) [463]. IgGs that bind to FcR in the endosomes of endothelial cells are protected from catabolism and are released back to circulation, while IgG that does not bind to FcRn passes to the lysosome to be degraded [464]. As shown in **Figure 17**, this therapy aims to block with specific monoclonal antibodies the FcR and to prevent binding of autoantibodies and induce their degradation.

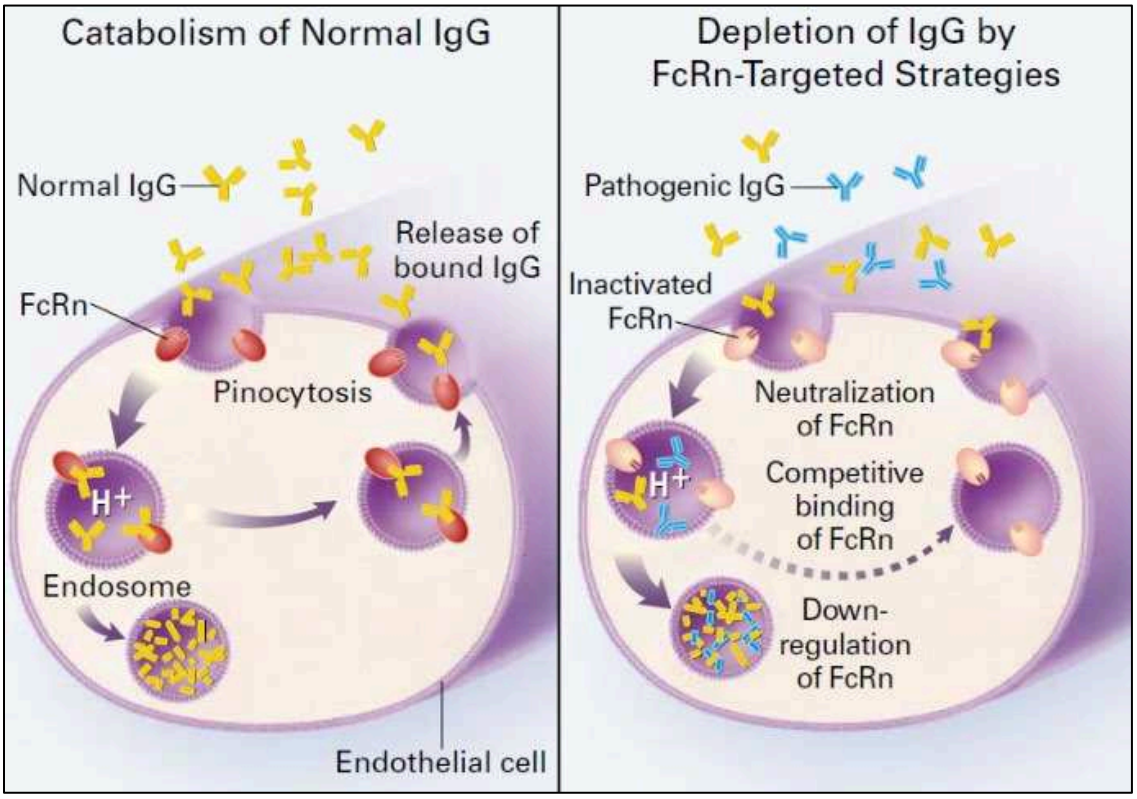

Figure 17 : Mechanism of action of anti-FcRn

An emerging therapy for antibody-mediated AIDs such as myasthenia gravis is neutralization of neonatal Fc receptor. This therapeutic approach is based on neutralization with monoclonal antibodies that prevent the capture of IgGs by FcRn and induce the destruction of potentially pathogenic antibodies in the endosome of endothelial cells. Adapted from [463].

Besides FcRn, most of the ongoing clinical trials are either therapies already widely used for the treatment of MG, such as mycophenolate mofetil, azathioprine and acetylcholinesterase, or recently accepted therapies that target the complement pathway or B-cells and focus on patients with refractory MG.

There is evidence that the IL-23/Th17 pathway plays a critical role in the development of autoimmune diseases (including MG, as I have demonstrated in this thesis). Surprisingly, therapies with monoclonal antibodies that target IL-17, IL-12p40, IL-23p19 or even IL-6R can be effective in pathologies like psoriasis and rheumatoid arthritis but have never been tested in MG. Nevertheless, in MG not many studies have shown explicit and convincing evidence of the implication of this pathway in the targeted tissue (muscle) or the effector tissue (thymus) of AChR^{+} MG.

4. Thesis aims

Myasthenia gravis is a neuromuscular disorder caused mainly by the presence of autoantibodies against the α-subunit of the acetylcholine receptor at the neuromuscular junction. While the clinical symptoms are focalized in the muscle, the genesis of the pathology takes place in the thymus.

The AChR$^+$ MG thymus harbors an overexpression of chemokines such as CXCL13, CXCL2 and CCL21 that promote the abnormal infiltration of B-cells [403, 404]. Moreover, the MG thymus presents a pro-inflammatory microenvironment. On one side, thymic AChR$^+$ MG regulatory T-cells present a reduced suppressive capacity and an abnormal overexpression of IL-17 and IL-21 [417]. On the other side, the AChR$^+$ MG thymus has an increased production of cytokines IL-6, IL-1β and TGF-β1 [93, 418, 419], cytokines that promote the initial development of pro-inflammatory T-cells (i.e., Th17 cells). Converging recent studies suggest that Th17 cells may play a role in the maintenance of the peripheral blood and thymic inflammatory environment. Th17 cells also contribute to maintain the GCs that promote differentiation of B-cells and the production of antibodies.

In physiological conditions, Th17 cells are present in barrier tissues in homeostasis with local microbiota and control infections by extracellular pathogens. However, loss of homeostasis can induce chronic inflammation that promotes an abnormal development of Th17 cells. Th17 cells are now recognized as drivers of autoimmune diseases. Development of Th17 cells relies on a combination of cytokines (IL-6, IL-1, TGF-β and IL-23). Of those, IL-23 has been demonstrated to be a “game changer.” Presence of IL-23 in the differentiation of Th17 cells promotes a pathogenic phenotype capable of inducing autoimmune diseases [1, 121, 197, 465]. These observations have led to the development of therapies that aim to control the effects of Th17 cells on AIDs. These therapies take advantage of the development of highly effective monoclonal antibodies that target cytokines or cytokine receptors related to Th17 cells.

To date, no curative therapy is available for autoimmune MG. To date, treatment of MG patients depends on glucocorticoids and immunosuppressors. Although some patients improve with these therapies, the therapies do not provide a cure and have many adverse effects. Other therapies such as anticholinesterases, plasma exchange and IVIg treat only symptoms and do not aim to control the immune deregulation. Thymectomy is the most effective therapy, but it is not curative. Therefore, development of new therapeutic approaches to treat MG, especially at the immune-pathological process in the thymus, must be developed.

The first objective of my doctoral project was to decipher at the cellular and molecular levels the role of Th17 cells in sustained inflammation in the thymus and serum of AChR$^+$ MG patients. To achieve this objective, we characterized by flow cytometry the cells producing IL-17 in AChR$^+$ MG thymuses. We then determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry whether MG thymuses expressed cytokines required for Th17 cell development. We investigated with in vitro experiments and purified cell subtype analyses the potential thymic cells producing IL-23. Finally, we established a coculture system to determine the impact of cytokines produced by mTEC on Th17 cell differentiation.

The second objective of my project was to investigate the impact of monoclonal antibody therapy that targets Th17 cells (i.e., anti- IL-23) on the thymic inflammatory events and clinical symptoms in a humanized MG mouse model and to address their mechanisms of action. To achieve this objective, we treated NSG thymic-engrafted mice with an anti-IL-23p19 monoclonal antibody. We determined in the NSG-MG mouse model whether treatment with anti-IL23p19 is capable of reducing 1) the thymic inflammatory process and 2) the peripheral inflammation (blood and spleen). The possibility that this model offers for recovering MG thymus after engraftment allowed us to determine the effect of anti-IL-23p19 therapy on the development process of Th17 cells (cytokine expression), on Th17 activity through their cytokine secretion, and on the stability of the eGC, structures of development and maturation of antibodies producing B-cells. We also evaluated the development of myasthenic symptoms.

The third objective of my Ph.D. project was to explore the impact of monoclonal antibody therapy on molecular changes and on satellite cell biology in skeletal muscle in the classical experimental autoimmune MG mouse model. Therefore, we evaluated the effect of anti-IL-23p19 therapy on 1) the clinical myasthenic symptoms (clinical muscle behavior), 2) the production of antibodies, 3) muscle physiology and 4) on isolated muscle fiber function.

During this Ph.D. work, we have provided in vitro evidence of the implication of the IL-23/Th17 pathway and the possible mechanisms of retro-positive control between mTEC and Th17 cells in the MG thymus. Finally, taking advantage of two MG mouse models, we showed that a therapy with an anti-IL-23p19 monoclonal antibody is capable of reducing thymic inflammation and reducing the production of anti-AChR antibodies, which translates into fewer clinical symptoms.

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II. RESULTS

Article #1

Cultured Human Thymic-Derived Cells Display Medullary Thymic Epithelial Cell Phenotype and Functionality

Objective

Thymic epithelial cells (TEC) are a heterogeneous cell population that encompasses two main types of cells based on their localization in the thymus: cortical TECs (cTECs) and medullary TECs (mTECs). mTECs play a key role in T-cell differentiation, maturation and education and have been widely investigated in mouse models. However, these models may not be used to decipher and investigate the human mTEC-specific features. More, in the context of myasthenia gravis disease, preliminary data have suggested a potential active role of mTECs in the disturbed thymic events occurring in $AChR^{+}$ MG patients. Therefore, to understand and to investigate the MG mTECs, we first analyzed the characteristics and features of primo-cultured human TECs. We then characterized the nature of the cultured cells from their morphology, their ability to express various factors (cytokines, chemokines, transcription factors and tissue-specific antigens), and their ability to respond to various stimuli to their functionality.

As we demonstrated, the primary cultured human TEC is an effective model that will allow investigation of the details of human mTECs in normal or pathological conditions.

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METHODS
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Cultured Human Thymic-Derived Cells Display Medullary Thymic Epithelial Cell Phenotype and Functionality

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Thymic epithelial cells are one of the main components of the thymic microenvironment required for T-cell development. In this work, we describe an efficient method free of enzymatic and Facs-sorted methods to culture human medullary thymic epithelial cells without affecting the cell phenotypic, physiologic and functional features. Human medulla thymic epithelial cells (mTECs) are obtained by culturing thymic biopsies explants. After 7 days of primo-culture, mTECs keep their ability to express key molecules involved in immune tolerance processes such as autoimmune regulator, tissue-specific antigens, chemokines, and cytokines. In addition, the cells sensor their cultured environment and consequently adjust their gene expression network. Therefore, we describe and provide a human mTEC model that may be used to test the effect of various molecules on thymic epithelial cell homeostasis and physiology. This method should allow the investigations of the specificities and the knowledge of human mTECs in normal or pathological conditions and therefore discontinue the extrapolations done on the murine models.

Keywords: thymic epithelial cells, primary cell culture method, keratins, cytokines, chemokines, tissue-specific antigens

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INTRODUCTION

The thymus is a primary lymphoid organ where T cell development and maturation take place. The thymic stromal cells correspond to heterogeneous cell types including mainly thymic epithelial cells (TECs) but also dendritic cells (DCs), macrophages, myoid cells, and fibroblasts. They create a tridimensional network to establish close contact with T-cells to support and to direct T-cell differentiation, maturation, and selection (1–3).

Derived from a common progenitor (4–6), two types of TECs based on their localization in the thymus are defined: medulla thymic epithelial cells (mTECs) and cortical thymic epithelial cells (cTECs) (7). cTECs contribute to the T cell lineage commitment and the positive selection, processes

Abbreviations: α-AChR, α-acetylcholine receptor; Aire, autoimmune regulator; BSA, bovine serum albumin; cTEC, cortical thymic epithelial cell; DC, dendritic cell; DHT, dihydrotestosterone; FBS, fetal bovine serum; Fezf2, Fez family zinc finger protein 2; GAD67, glutamic acid decarboxylase 67; HBSS, Hank's balanced salt solution; IL-6, interleukin 6; IL-1β, interleukin 1β; K5, keratin 5; K8, keratin 8; K14, keratin 14; K18, keratin 18; Lt-β, lymphotoxin-β; mTEC, medulla thymic epithelial cell; NKT, natural killer T cell; PBS, phosphate-buffered saline; PLP, myelin proteolipid protein; Prdm1, PR domain zinc finger protein 1; Rankl, receptor activator of nuclear factor kappa-B ligand; TGF-β, tumor growth factor-β; TG, thyroglobulin; Treg, regulatory T cell; TNFR, TNF receptor; TSA, tissue-specific antigen; UEA, *Ulex europaeus* agglutinin-1.

that allow the development of T cells exhibiting specific T cell receptor with low or high affinity to MHC molecules (8). mTECs complete and finalize T cell maturation processes through the negative selection of T cells as well as the generation of regulatory T cells (Tregs) and natural killer T cells (NKT) (9–11). To ensure their role in immune tolerance, mTECs express a wide range of proteins that correspond to self-antigens expressed by peripheral tissues and organs. The expression of these tissue-specific antigens (TSAs) in mTECs is regulated by specific transcription factors: autoimmune regulator (AIRE), Fez family zinc finger protein 2 (FEZF2), and PR domain zinc finger protein 1 (Prdm1) (12, 13). As a consequence, in mice with mutations in these genes, mTECs are defective in their tolerance process and mice are associated with autoimmune phenotypes. In addition, mTECs express various cytokines [interleukin 6 (IL-6), interleukin 1β (IL-1β)], and chemokines (CCL19 and CCL21) (14–17) that are critical for the T cell signaling and migration throughout the cortex/medulla compartment. Therefore, alterations in mTECs are deleterious for T cell differentiation and can lead to complete blockage of thymopoiesis (18) or reduce thymocyte proliferation (19), as illustrated in thymuses of knockout mice for FOXN1, and CBX4. Foxn1 is a transcription factor that regulates, during the thymus development, the expression of CCL25, Dll4, and Hoxa3, factors required for the thymocyte and TEC differentiation (20). Furthermore, CBX4 is a crucial and non-redundant protein that controls the generation and maintenance of the thymic epithelium (19).

The functional role of TECs has been studied using well-established mouse models (21–23) while few human models are available (24–29). These numerous models reveal murine mTEC features and functions, but they cannot apply to human mTEC specificities. Indeed significant steps are characterized by dissimilar kinetic expression of specific markers in humans versus mice (30). So far, few models are developed in humans. Fernandez et al. have characterized cloned TEC lines derived from human cortical epithelium (31). In addition, Patel et al. have found that TECs obtained from cultured human explants (32), display similarities to keratinocytes compared with others cell types by screening membrane markers. An enzymatic procedure to isolate and then to culture human primary TECs has been set up (33) and has later been characterized by Skogberg et al. (34). However, it is reported that enzymatic digestion can affect expression level of surface molecules (35) and then influence the purification process and the cell viability (36). To avoid these issues, here we describe an efficient enzyme-free procedure to culture primary human mTECs by using fresh thymic explants. This method allows in a reduced processing time and with a limited tissue amount, to obtain a significant number of cells displaying mTEC features and expressing specific TEC surface markers and TSAs. This model is a viable cell model indispensable to investigate human mTEC physiology in normal or pathological conditions.

MATERIALS AND METHODS

Human Samples

Human thymic fragments (5–10 g) were obtained from immunologically normal male and female newborns (age 2 days to 1-year old) undergoing corrective cardiovascular surgery at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France). To perform RNA and protein analyses, tissue samples were fast frozen in liquid nitrogen. To establish primary human thymic epithelial cells (TECs), pieces of tissue were placed in sterilized RPMI medium and maintained at 4°C until cell culture procedure.

This study was under the French Bioethic Law that requires a written informed consent from the donors or the legal representant. In respect to this law, this study was approved by the local ethics committee (CPP, Kremlin-Bicêtre, France: agreement No. 06-018; CCP Ile de France Paris 7, France agreement No. C09-36).

Primary Cell Cultures

Primary human TEC cultures were established on the basis of previous reports (11, 37, 38). Human thymic tissues were cut with scissor into small fragments (~10 mm^2) in Hank's balanced salt solution (HBSS) medium and then washed three to four times in HBSS medium to discard most of thymocytes. The washed thymic explants were transferred to 75-cm^2 culture dishes and were allowed to attach to the flask for 5 min without medium. Ten ml of fresh culture medium were added gently and flasks were placed in a chamber incubator at 37°C with 5% CO_2. The culture medium was prepared with RPMI 1640 supplemented with 20% horse serum (Life Technologies, Cergy-Pontoise, France), 0.2% Ultroser G (Biosepra, Cergy, France), 2 mmol/l of L-glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (Life Technologies, Cergy-Pontoise, France). Twice a week, the medium was removed, the cells were washed once with phosphate-buffered saline (PBS) 1× (Life Technologies, Cergy-Pontoise, France), and a fresh culture medium was added. Rapidly, cells migrated out of the thymic explants and expanded around the biopsies. After 7–8 days, cells were washed with PBS, and a first trypsin treatment [0.075% trypsin (Life Technologies, Cergy-Pontoise, France) and 0.16% EDTA (Life Technologies, Cergy-Pontoise, France)] was performed during 3 min to remove fibroblasts that could have grown in the cultures. Primary human TECs remained attached to the flasks, and then they were rinsed with PBS. A new trypsin solution was added, and TECs were collected by adding 5 ml of 0.075% trypsin solution for 5 min at 37°C. The content of each flask including detached cells and explants was filtered using 40 µm cell strainer (Dutscher, Brumath, France). Then, the explants were removed, and the individual cells were diluted in culture medium devoid of Ultoser-G and supplemented with 5% horse serum. The nature of TECs and the percentage of medullary TECs were analyzed as described below before being seeded at a density of 2–3 × 10^3 cells into 12 or 24-well Nunc cell plates (Life Technologies, Cergy-Pontoise, France). Cells were allowed to attach to the plates for 24 h before being treated for 24 h.

Thymocytes were isolated from human thymi by mechanical dissociation of fresh thymic tissue, as previously described (39, 40). The cells were filtered through cell strainer advice to remove thymic tissues and washed once with HBSS. Thymocytes were quick frozen upon RNA extraction.

Fibroblasts were obtained after 7 days of thymic explants culture during the first trypsin step as previously described by Cufi et al. (41).

Immunofluorescence Microscopy

Cryostat sections (7 μm) of frozen thymic tissues and primary cultured TECs were fixed with acetone to glass superfrost slides and lab-teck chamber slides, respectively, and dried for 1 h. The thymic sections and primary cultured TECs were pre-incubated with a blocking buffer (PBS 1×, 0.1% bovine serum albumin, 10% fetal bovine serum, 0.3 M glycine, and 1% Tween) for 1 h at room temperature and, then incubated for 2 h at room temperature with antibodies raised against human antigens listed in Table S1 in Supplementary Material. The labeled cells were revealed with Alexa 488- and/or Alexa 594-coupled secondary IgG raised in donkey or chicken, respectively. Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope using 20× magnification (Carl Zeiss, Le Pecq, France).

At day 7 of culture, the nature of TECs and the percentage of medullary TECs were estimated by flow cytometry and immunofluorescence using MNF116 antibody (Dako, Trappes, France) and an anti-keratin 5/14 antibody (Covance, Rueil-Malmaison, France) (42, 43), respectively. The MNF116 antibody is a pan-cytokeratin antibody that recognizes various cytokeratins 5, 6, 8, 17, and 19 (K5, K6, K8, K17, and K19). The anti-keratin 5/14 antibody recognizes the medullary TECs on thymic sections (44). An anti-collagen III antibody (Clone III-53; ICN) was used to identify fibroblast cells. Under a percentage of 80% of TEC cells, cells were discarded because the presence of other contaminating cells, mainly fibroblasts (Figures S1 and S2 in Supplementary Material).

RNA Extraction

Total RNA was prepared from the thymus and TECs using the trizol RNA Isolation kit (Life Technologies, Cergy-Pontoise, France). Thymuses were homogenized with the FastPrep FP120 instrument (Qbiogen, Illkirch, France). The concentration of RNA was analyzed with a NanoDrop ND-1000 spectrophotometer (LabTech, Palaiseau, France). RNA samples presenting a minimal ratio of 1.9 and 2 for, respectively, 260/280 and 260/230 were also controlled on a gel. The RNA quality was assessed with the ARN FlashGel™ System. When the samples were degraded even partially, they were excluded.

Real-Time PCR

The reverse transcription of total RNA into cDNA was performed by using the SuperScript II RT kit (Life Technologies, Cergy-Pontoise, France) according to the manufacturer's instructions. PCR reactions were completed using the LightCycler apparatus as previously described (38). The primers used for the real-time PCR are listed in Table S2 in Supplementary Material. Each cDNA sample was run at least in duplicate.

Flow Cytometry

Flow cytometry analysis of the MNF116 expression in primary cultured TECs was done according to the protocol described by the manufacturer. MNF116 antibody recognizes cytokeratins (5, 6, 8, 17, and 19). Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% PBS-Tween. The permeabilized cells were incubated with either an uncoupled mouse anti-MNF116 antibody (Dako, Trappes, France) or uncoupled mouse anti-collagen III antibody (ICN Biomed, Illkirch, France) and washed twice with PBS 1×. MNF-116 or collagen III staining was detected with Alexa 488 coupled chicken anti-mouse IgG (Life Technologies, Cergy-Pontoise, France). After two washings with PBS, cells were analyzed on the FACSVerse apparatus using the Facs suite software (Becton Dickinson, Le Pont-de-Claix, France).

Statistical Analysis

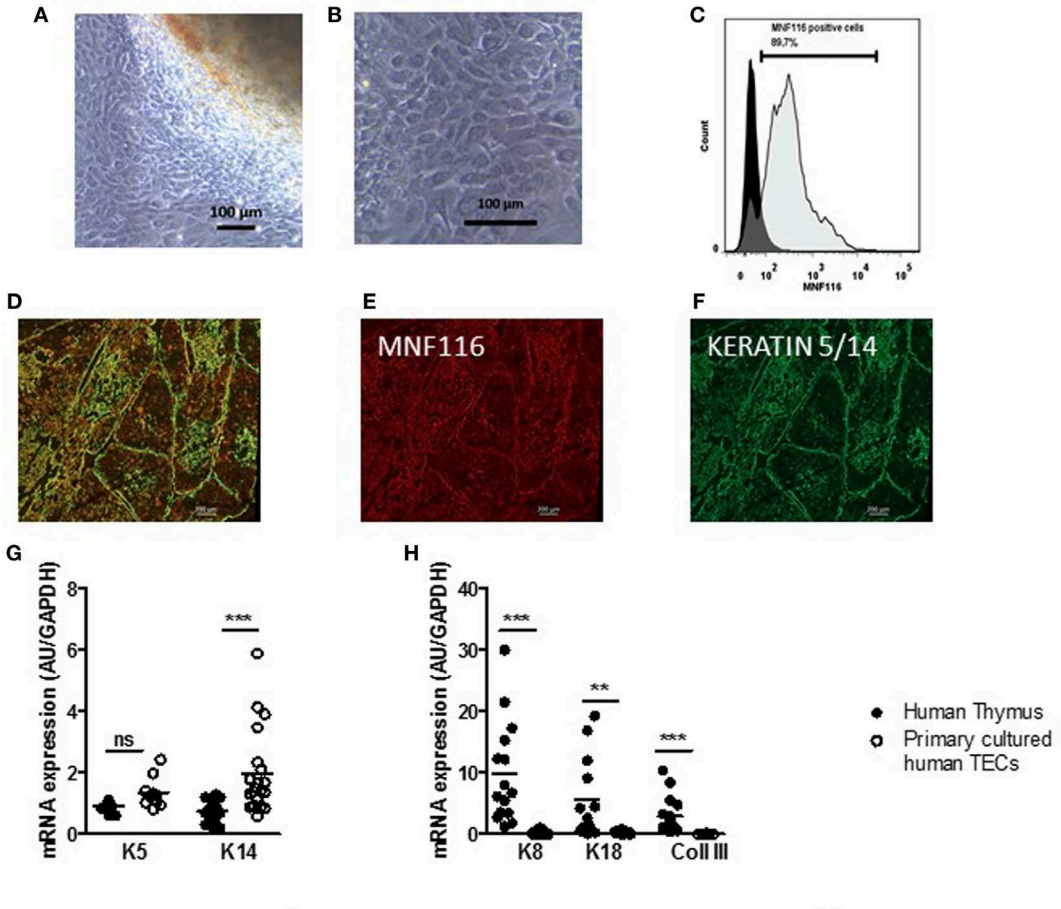
ANOVA and Mann–Whitney tests were used as specified in each figure. Statistical significance is recognized at $p < 0.05$. GraphPad prism was used for all the statistical analysis.

RESULTS

Human Thymic-Derived Cells Display mTEC Morphology and Surface Markers

Cultures of human thymic explants could lead to heterogenous cultures of thymic cells. However, our culture conditions mainly allowed the proliferation and survival of thymic stromal cells, since non-adherent cells namely the thymocytes remain in the supernatants and are eliminated during washing steps. After 2–4 days, the primary thymic cells expanding from the thymic explants (**Figure 1A**) displayed closely packed cells that harbored the morphologic mosaic form of epithelial cells (**Figure 1B**). The cultured cells stained positively to MNF-116 (**Figure 1C**), a pan-cytokeratin antibody that recognizes various cytokeratins 5, 6, 8, 17, and 19 (K5, K6, K8, K17, and K19) expressed in thymic medullary and cortical epithelial cells. As observed in **Figures 1D,E** on thymic sections, the MNF116 antibody labeled the entire thymic epithelial network (medulla and cortical areas), while the anti-K5/K14 antibody stained specifically the thymic medulla area (**Figure 1F**). These data suggested that whether primary cultured thymic cells display morphological and phenotypical appearance of TECs using the MNF116 antibody did not discriminate cTECs from mTECs in thymic-derived cell cultures. Nevertheless, inherent to the thymic explants, cell populations other than TECs could be found in low proportion as shown in Figure S1 in Supplementary Material. At day 7–8, when fibroblast cells considered as contaminants, exceeded 20%, the culture was discarded. Fibroblasts were identified by staining with anti-collagen 3 antibody (Figure S2 in Supplementary Material).

To further characterize TECs, at day 7 of culture, we quantified by qPCR the level of expression of various TEC markers in thymic biopsies and the primary cultured TECs (**Figures 1G,H**). We showed that thymic biopsies expressed mainly cortical (*K8/K18*) and fibroblast markers (**Figure 1H**). By contrast, primary human TECs expressed mainly medullary markers (*K5* and *K14*) (**Figure 1G**); cortical keratins (*K8* and *K18*) and fibroblastic marker (*COLLAGEN III*) expressions were barely detectable (**Figure 1G**). By using the microdissection technology on human thymus sections, we showed that 82% of *K5* thymic expression and 87% of *K14* thymic expression were due to the medullary microdissected areas of human thymuses while K8 was mainly cortical



I

	Thymus	TECs	TEC enrichment ratio (TECs /Thymus)
K5	0,9	1,4	**1,50**
K14	0,7	2,0	**2,64**
K8	9,9	0,3	**0,03**
K18	5,6	0,4	**0,07**

FIGURE 1 | Primary cultured human thymic stromal cells display thymic epithelial cell (TEC) features. **(A,B)** Representative pictures of thymic cells expanding from human thymic explants. **(A)** After 2–4 days of culture, cells migrate and proliferate out of the explants in culture. **(B)** Thymic cell culture with the morphologic mosaic form of epithelial cells. **(C)** Representative flow cytometry plot of primary cultured TECs labeled with an anti MNF-116 antibody; the control isotype is shown in black at a day 7. **(D)**. Representative pictures of primary human thymic sections **(E–G)** co-labeled with an anti-MNF-116 antibody (red) **(E)**, and anti-keratin 5 and 14 antibodies (green) **(F)**. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope. mRNA expression levels of medulla thymic epithelial cell (mTEC) markers (*K5* and *K14*) **(G)**, cortical thymic epithelial cell (cTEC) markers (*K8* and *K18*), and a fibroblast cell marker (collagen III, *Coll III*) **(H)** in human thymuses and in primary cultured TECs (day 6–7). Enrichment ratios of gene expression of specific markers for mTECs (gray) and cTECs (blue) in cultured human TECs (day 7) versus total thymus **(I)**. mRNAs were analyzed by real-time PCR and normalized to GAPDH for total thymuses and primary TECs. mRNAs are expressed as arbitrary units and are the mean values ($n > 9$ for thymuses and $n > 5$ for different donors for primary cultured TECs). p Values were obtained using the ANOVA test. Asterisks indicate significant differences (**$p < 0.005$ and ***$p < 0.0001$).

(Figure S3 in Supplementary Material). The compared analysis of the gene expressions and their ratios in TEC cultures versus thymic biopsies, confirmed that our culture method sustained the growth of cells expressing predominantly medullary markers such as *K5* and *K14*, with an enrichment ratio between 1.5 and 2.64 while it was below 0.03 for cortical markers (**Figure 1I**).

Medulla thymic epithelial cells are a heterogeneous cells composed of different cell subpopulation identified by functional, phenotypic, and developmental markers (45–47). We wondered whether this diversity is maintained in culture. To this end, we investigated the protein expression of various specific mTEC markers, such as CLAUDIN 4, CLAUDIN 3, tight junction components known to be expressed by mTEC lineage-committed cells and *Ulex europaeus* agglutinin-1 (UEA) lectin (27, 48, 49), a marker of highly proliferative mTECs expressing autoimmune regulator (AIRE) protein (45).

Figure 2 showed that cultured cells exhibited positive labeling for K5/14, for CLAUDIN 4 (**Figures 2A–C**) as compared with thymic biopsies (**Figures 2D–F**). These labeling mirrored the medulla compartment of the thymus tissue (**Figures 2D–F**). The UEA antibody labeled few cultured mTECs (**Figures 2G–I**). Similarly, few mTECs in human thymic sections were stained with this antibody (**Figures 2J–L**). The percentage of positive cells in cultured mTECs and in the thymic medullary areas is shown for the different markers in **Figure 2M**, and no statistical differences were observed. Altogether, these data showed that our culture model maintained a diversity of the mTEC subpopulations comparable with that in global thymuses.

Human Primary Cultured mTECs Express Factors Involved in T Cell Negative Selection Process

Medulla thymic epithelial cells play a major role in immune tolerance by expressing and presenting TSAs to developing T cells. TSAs expression in mTECs is controlled by various transcription factors among them AIRE, FEZf2, and PRDM1. We evaluated the ability of cultured primary TECs to express such tolerance markers. At day 7, we observed that primary cultured TECs expressed *AIRE*, *PRDM1*, and *FEZF2* (**Figure 3A**) and various TSAs, such as the α-acetylcholine receptor (α-*AChR*), thyroglobulin (*TG*), myelin proteolipid protein (*PLP*), and glutamic acid decarboxylase 67 (*GAD67*) (**Figure 3B**). The significant high expression of these various factors in primary cultured TECs compared with the thymic tissue highlighted that analysis of gene expression in global tissue (which is a mix of various cell types) may underestimate the gene expression in a cell type. More, these data suggested that cultured mTECs are probably functional since they remain able to express these specific functional markers. For α-AChR, the gene expression ratio between cultured TECs and total thymus did not display an increase. This is in part due to the fact that in the thymus, the AChR subunits are highly expressed in myoid cells (muscle-like cells) compared the TECs (51).

Human Primary Cultured TEC Express Cytokines and Chemokines

Medulla thymic epithelial cell involvement in T cell development and maturation is not exclusively based on negative selection and tolerance process. To help T cells during their long journey across the thymus, mTECs secrete "guiding molecules" such as cytokines and chemokines that play a crucial role in T cell migration or signaling, and that could, in turn, affect TEC differentiation (52). We compared the expression level of cytokines and chemokines in our primary cultured TECs with other thymic cells (thymocytes and fibroblasts) and with thymic biopsies. We observed that cultured human primary TECs displayed a significant high mRNA expression of *IL-6* (**Figure 4A**), tumor growth factor-β (*TGF-β*) (**Figure 4B**), *CCL21* (**Figure 4C**), and *CCL19* (**Figure 4D**) compared with the other cell types. Of course, in human thymuses, different cell types may express *IL-6*, *TGF-β*, *CCL21*, and *CCL19*. However, in normal conditions, TECs appeared to be the main sources of TGF-β, CCL21 and CCL19 while IL-6 can also be provided by fibroblastic cells. Therefore, the data demonstrate that, after 7 days of culture, mTECs still displayed their ability to express molecules promoting their own differentiation as well as migration of thymocytes, validating the quality and the potential functional efficiency of the primary human mTEC culture model.

Are Primary Cultured Human TECs Responsive to Stimuli?

It is well documented that estrogen targets mTEC transduction pathways, through its receptors (ER-α and ER-β) and then modulates the immune response (38, 53, 54). We have previously demonstrated that estrogen may modulate in mTECs, expression of AIRE and TSAs as well as cytokines and chemokines, such as IL-6 and CXCL13 (50, 55). More, the increased intrathymic level of inflammatory cytokines, such as IL-1α or IL-1β (27), during aging process, has been shown to affect TEC homeostasis and functionality (27). Consequently, primary TECs obtained from thymic explants were sub-cultivated and challenged with estrogen and IL-1β to evaluate their reactivity to stimuli.

First of all, we checked the gene expression induction capacity by the cultured mTECs. To this end, we added receptor activator of nuclear factor kappa-B ligand (RANKL) in the cell medium since *AIRE* mRNA expression is regulated by RANK/CD40 and lymphotoxin beta receptor signaling pathways (56–58). We observed a significant increase of AIRE mRNA expression (**Figure 5A**) suggesting that the cultured cells conserved their ability to overexpress AIRE upon stimulation.

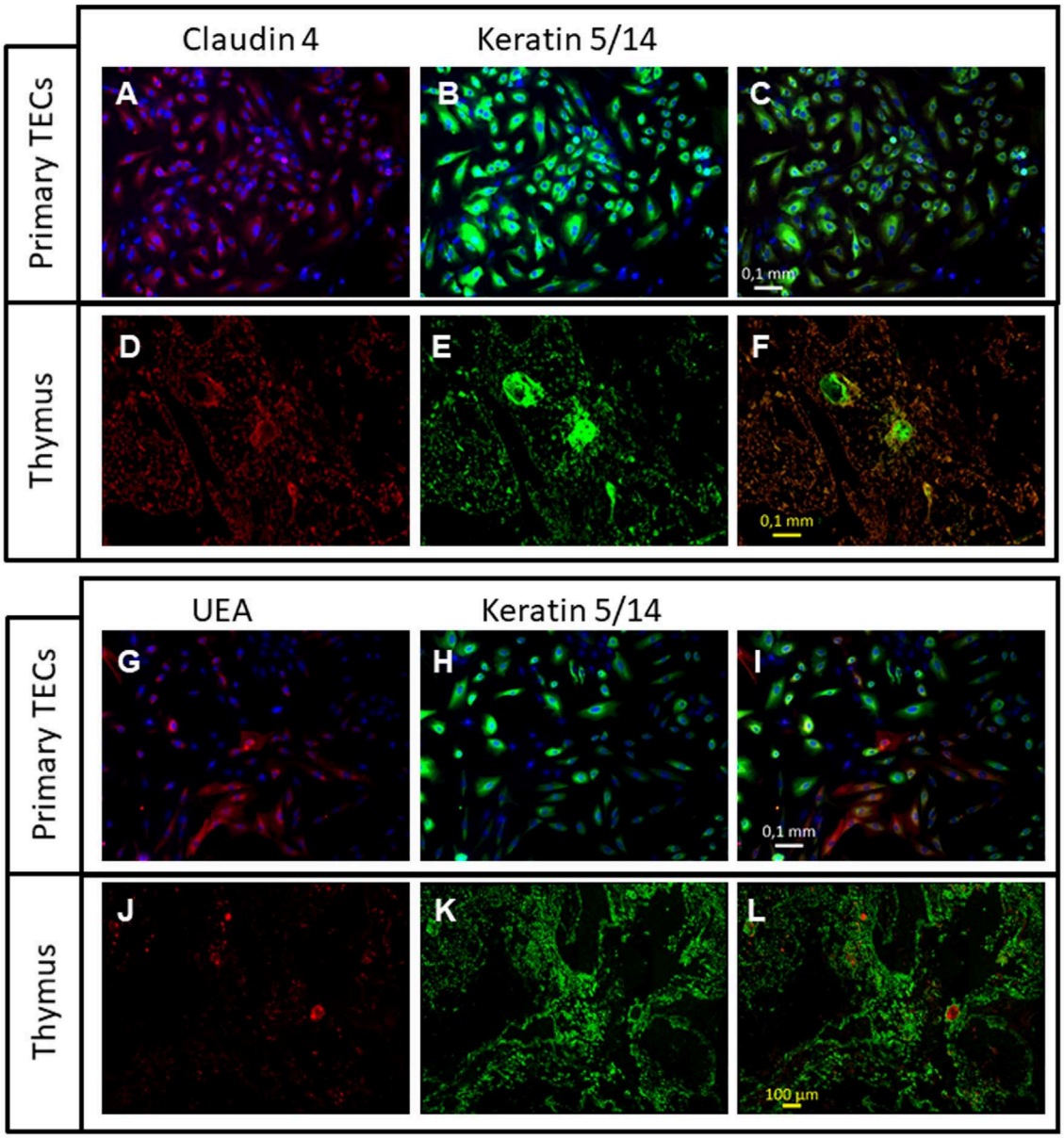
Therefore, cultured primary human mTECs were challenged with estrogen and the inflammatory cytokine IL-1β to investigate the *in vitro* cell responsiveness.

Our data showed that estrogen decreased *AIRE* expression (**Figure 5B**) in cultured mTECs, corroborating what have previously been shown (50). In addition, mRNA expression of *PLP* (an AIRE independent TSA) is significantly increased by estrogen (**Figure 5C**). More, we showed that the expression of the signaling molecule *IL-6* and *CCL21* is inhibited by estrogen while *TGF-β* mRNA expression remained unaffected. Altogether, these data suggest that estrogen activated in cultured human mTECs transduction pathways that regulated gene expression. Interleukin-1β induced no effect on *AIRE* expression (**Figure 5B**) while it inhibited mRNA expression of *PLP* (**Figure 5C**), highlighting that cultured mTECs display functional different transduction pathways (estrogen related network for AIRE, and inflammatory network for PLP) involved in the regulation of key tolerance molecules. More, IL-1β stimulated *IL-6* mRNA expression, and no effect was observed for *CCL21* and *TGF-β* mRNA expression (**Figures 5D–F**).

These data suggested that inflammatory and intrinsic factors modulate in primary cultured mTEC, expression of signaling molecules involved in T cell differentiation (IL-6 and CCL21) as well as in their own differentiation.

DISCUSSION

Here, we report that primary cultured of human thymic cells derived from human thymic explants, displayed mTEC features, remained functional to provide molecules for thymocyte education and maturation, and were able to respond to distinct specific



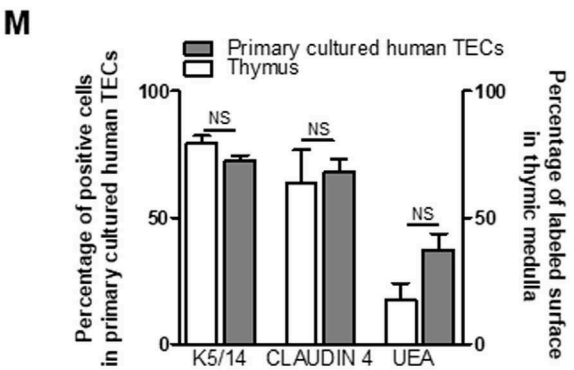


FIGURE 2 | Continued

FIGURE 2 | Primary cultured human thymic cells display medulla thymic epithelial cell features. Representative pictures of a primary cultured human thymic epithelial cells (TECs) (day 7) **(A–C)** and human thymus **(D–F)** co-labeled with an anti-Claudin 4 antibody (red), anti-keratin 5, and 14 antibodies (green). Representative pictures of primary cultured human TECs **(G–I)** and human thymus **(J–L)** co-labeled with an *Ulex europaeus* agglutinin I lectin (UEA) (red), anti-keratin 5 and 14 antibodies (green). The percentage of positive cells in primary cultured human TECs represented the number of KERATIN 5/14, CLAUDIN 4, or UEA positive cells versus the total cell number **(M)**. For thymic sections, the surface of KERATIN 5/14 or CLAUDIN 4 positive areas was measured and compared with the thymic medulla. Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope using 20× magnification. The counting was done as previously described in Dragin et al. (50). ImageJ software was used to display the digital pictures and to count manually the labeled cells. Graph bar represents the results obtained with four different human biopsies and primary cultured human TECs. The non-parametric Mann–Whitney test was used for statistical analyses.

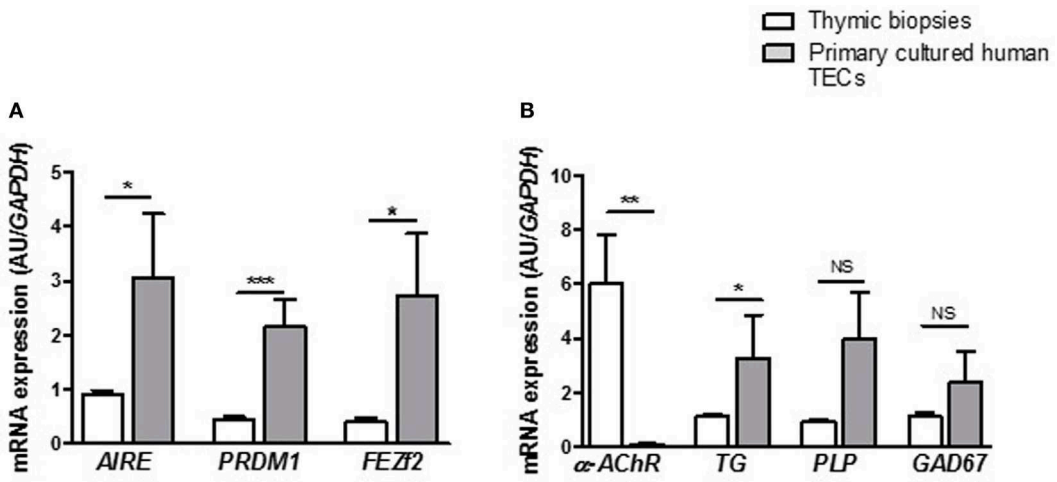


FIGURE 3 | Primary human thymic epithelial cells (TECs) express immune tolerance molecules. mRNA expression levels of transcription factors **(A)** and tissue-specific antigens **(B)** in human thymuses and in primary cultured TECs. mRNA expressions were analyzed in primary cultured TECs at day 7 of culture. mRNAs were analyzed by real-time PCR, normalized to *GAPDH*, and are expressed as arbitrary units (±SEM) ($n > 5$ for thymuses and $n > 5$ different donors for primary cultured TECs). p Values were obtained using the non-parametric Mann–Whitney test. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.007$, and ***$p < 0.0001$).

signals from their environment. Even-though cTECs cannot be studied with our cultured cell model and this model is a short term culture model (7–8 days) that will promote the establishment of disease-specific thymic epithelial cell lines that could be used in long-term analysis.

Most studies have used mouse models to decipher tolerance and T-cell differentiation processes. So far, combined mechanic and enzymatic procedures, to extract or to obtain TECs, have been reported (34). These procedures, commonly used in mouse, require a significant amount of thymus biopsies. In addition, enzymatic dissociation is often associated with FACS-sorted or bead-sorted methods to purify murine and human TECs (59–61). These methods have helped to decipher with omics analyses (transcriptome, miRnome, exome, and proteome) a fixed picture of mTECs reflecting their crosstalk and interaction with other thymic cells. However, these methods are not followed by prolonged cell culture time due to the reduced extracted cell number and to the decreased cell viability (60). Therefore, we have optimized a technique that reduces the required amount of tissue and at the same time preserves cell viability and increases the number of extracted cells. In addition, human T-cells display specificities that require appropriate models that take into account human specificities. To this end, the establishment of human TEC cultures is a substantial step to address questions specific to the human T-cell development and differentiation. We observed that TEC cultures were functional, as molecular changes could be found after cytokine or hormone triggering. Thus, our culture method is not only useful for characterization of the epithelial cells *per se*, their morphology and surface characteristics, but also for their maturation process, secretion, and antigen presentation. This model will provide data, in humans and not mice, on the direct interactions between T-lymphocytes and thymic stromal cells, and could be the basis of the establishment of disease-specific thymic epithelial cell lines. Such method is the first step in the development of immortalized human mTEC lines (from control to disease-specific cell lines such as Myasthenia gravis or Omenn syndrome), models that will allow the understanding of the mechanisms underlining TEC function, thymic senescence, and aging as well as disease pathophysiological events.

Human Viable Medullary TECs May Be Cultured From Human Thymic Explants

In our culture conditions, thymic-derived cells displayed specific mTEC markers (K5, K14, Claudin 4, and UEA-1) while cTEC markers were not detected (K8 and K18). Various groups have characterized a battery of markers such as cell membrane adhesion molecules (Claudin 4 and UEA-1), intracellular molecules (keratin 5/14), and transcription factors (Aire and FezF2) to identify mTECs. To this end, our TEC culture model provided cells that express these mTEC markers with a significant expression

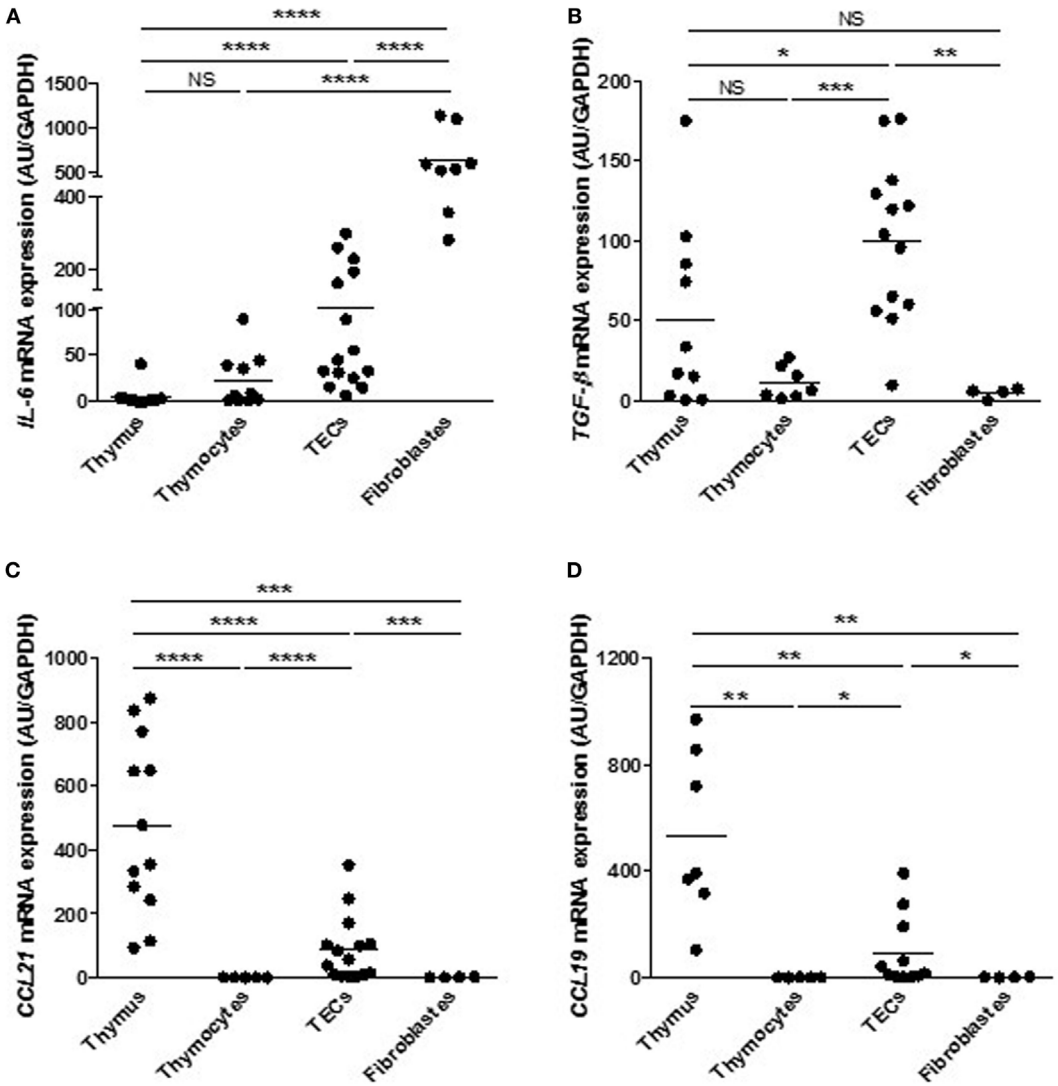


FIGURE 4 | Primary cultured human thymic epithelial cells (TECs) express cytokines and chemokines. Comparison of mRNA levels of interleukin 6 (*IL-6*) **(A)**, tumor growth factor-β (*TGF-β*) **(B)**, *CCL21* **(C)**, and *CCL19* **(D)** in different thymic cell types. mRNAs were analyzed by real-time PCR and normalized to *GAPDH*. For each gene, mRNA expressions in primary TECs were normalized to 100 and compared with others cell types. mRNA expression levels are expressed as arbitrary units and are the mean values ($n > 6$ for thymuses and $n > 4$ different donors for primary cultured TECs (day 7), fibroblasts or thymocytes). *p* Values were obtained using the Mann–Whitney test. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

level. Still, whether these markers characterize thymic cells as mTECs (49), they do not define accurately the mTEC development and differentiation state (48, 62). For instance, most studies agreed that Claudin 3 and 4 are expressed by a subset of mTECs that co-expressed the UEA marker (49). But, the period of expression of such markers from mTEC progenitor through mature mTEC stage diverged between groups (48, 49, 62). Though some consensuses have emerged such as mTEC differentiation stages are characterized by differential MHC II, CD80 expression levels (63) while Aire expression is often associated with mature mTECs (30, 43, 63, 64).

Furthermore, mTECs development and differentiation depend upon activation of lymphotoxin β-receptor and TNF receptor (TNFR) (65). TNFR is activated by CD40L and RANKL, two cytokines produced in the thymus by lymphoid cells (45, 66, 67). These transduction pathways also regulate AIRE, FEZF2, and PRDM1 expressions. As already observed and reported, in the primary cultured human mTECs, *AIRE* expression fluctuates during the culture process to reach its optimal expression around day 6–7 and then decline throughout the culture time (Figure S4 in Supplementary Material). Therefore, we can envisage that our cultured thymic explants provide signals to stimulate mTEC proliferation and differentiation through a certain period of time. Then, after 1 week, our culture method is devoid of the required lymphoid cells. A comparable observation was done for *AIRE* expression in another model of TEC culture (34) and in different murine TEC cultures (68, 69). In addition, gene decreased expression occurred quite often

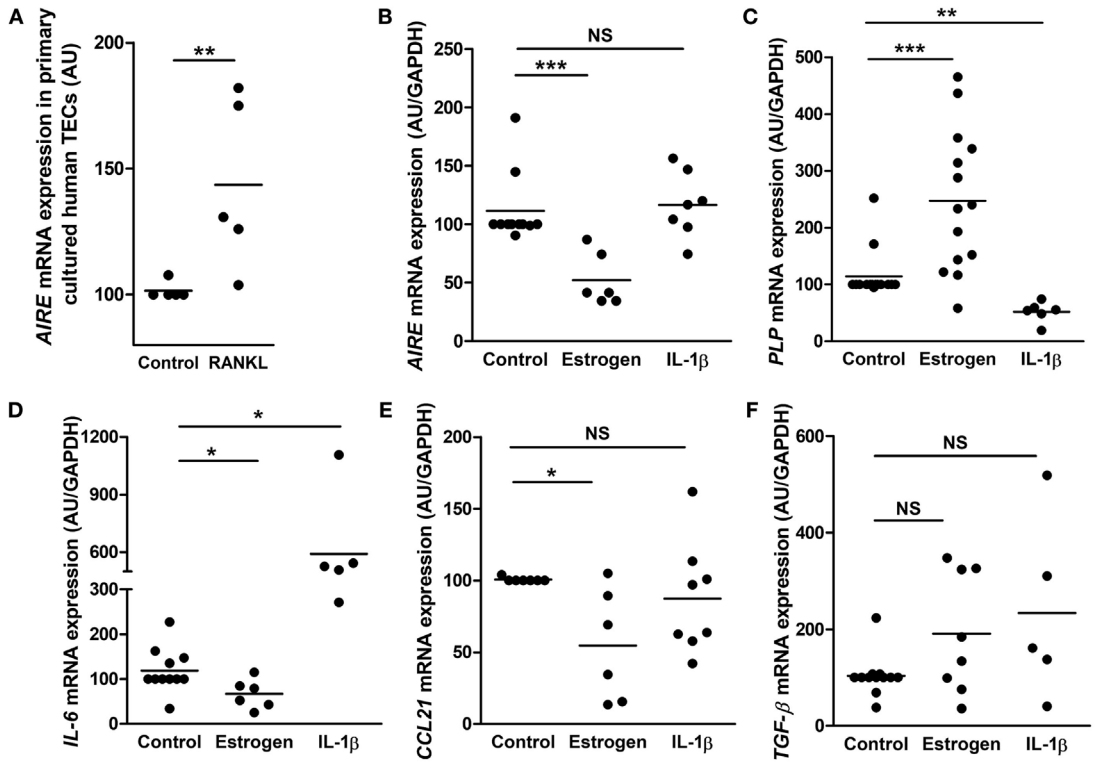


FIGURE 5 | Effect of estrogen and interleukin 1β (IL-1β) on gene expression in primary cultured human thymic epithelial cells (TECs). Effect of RANKL (10^{-9} M) on *AIRE* mRNA expression **(A)**. Effect of estrogen (10^{-8} M) or IL-1β (1 ng/ml) on the mRNA expression of *AIRE* **(B)**, myelin proteolipid protein (*PLP*) **(C)**, interleukin 6 (*IL-6*) **(D)**, *CCL21* **(E)**, and tumor growth factor-β (*TGF-β*) **(F)** in primary cultured human TECs. For each experiment, cells were incubated for 24 h. mRNA expressions were normalized to 100 in control untreated cells. Each point represents the mean value of an experiment using primary cultured TECs (day 7) obtained from different donors ($n > 5$). mRNAs were analyzed by real-time PCR and normalized to *GAPDH*. *p* Values were obtained using the non-parametric Mann–Whitney test. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

and is well documented for other proteins such as MHC II (70) or Fas (39), illustrating that primary cultured cells can lose throughout the culture time their ability to express specific factors, likely due to the depletion of specific cell types. In support to this hypothesis, the addition of RANKL, mainly produced by a $CD4^+$ T cell subsets, restored the expression of *AIRE*, validating that the decline of expression for certain genes is probably due to the lack or decrease of a thymic specific microenvironment (1). FOXN1 is required to induce both cortical and medullary thymic epithelial cell differentiation (7, 20, 71, 72). Recently, O'Neill et al. (73) have suggested that "Post-AIRE expressing TECs, a potential distinct stage of terminal mTEC development, are sub-functional TEC resulting from downregulation of *Foxn1* and MHCII" (73). To confirm this hypothesis, our culture model may be an appropriate tool that will clarify human mTEC differentiation process.

Human Derived mTECs Remain Keys Actor in T-Cell Maturation

Here, we demonstrated that cultured human primary mTECs do not lose their ability to provide signal molecules required for central tolerance basic mechanisms. A key hallmark of mTECs is their propensity to express TSAs to establish self-tolerance. AIRE, FEZF2 and PRDM1 are transcription factors identified to promote TSA expression in mTECs (13, 57, 63). In the thymus, where their expression is restricted to mTECs, they cooperate and do not displayed redundant roles as they modulate expression of different sets of TSAs in mTECs (13, 57, 74). Their essential roles, in negative selection or tolerance process, are highlighted in knockout mouse lines that develop autoimmune diseases with cell infiltrations in various peripheral tissues and autoantibody production (13, 57, 74).

In addition, mTECs secrete cytokines, chemokines, and growth factors (TGF-β, IL-6) molecules essential to maintain the T cell phenotype and to direct the differentiation and maturation in specific T cell subsets (65, 75, 76). In thymic medulla, TGF-β is mainly express by mTECs and in an autocrine manner regulates negatively the number of mTECs (77). TGF-β induces the expression of Foxp3, the Treg transcription factor in single positive $CD4^+$ T cells (78). Therefore, TGF-β plays an important role on the differentiation and survival of thymic Treg cells (11) and reduces the escape or proliferation of autoreactive T cells from the medulla to the periphery (77). TGF-β may act in combination with IL-6 to stimulate the expression of retinoid-related orphan receptor, specific transcription factor

of pro-inflammatory Th17 cells (79) in $CD4^+$ T cells. IL-6, a pro-inflammatory cytokine, is produced mainly by thymic stroma cells (fibroblasts and TECs) (80) and is implicated in the differentiation of $CD4^+$ T cell subsets and mediates B- and T-cell survival (81).

More, primary cultured human mTECs conserved their expression of chemokines, signal molecules mandatory to guide maturing T cells. Thus, *in vivo*, mTECs secrete several chemokines (CCL19, CCL21, CCL22, etc.) that attract T cells or DCs in the medulla during normal T cell maturation and migration throughout the thymus (82, 83). The migration of $CD4^+$ T cells to the medulla is operated by an active chemotaxis process through the CCR7/CCR4, receptors expressed by $CD4^+$ T cells (52). The sustained migration potentiates the interaction of differentiating thymocytes with thymic stromal cells, a process indispensable for their optimal differentiation and to perform the negative T cell selection (52, 84).

CONCLUSION

We demonstrated that, similarly to *in vivo* situations, thymic-derived mTECs could respond with an appropriate answer to estrogen and inflammatory signals. TEC fate is a combination of crosstalks with T cells and the environment. Human mTECs in culture maintain their ability to express specific cytokines and chemokines, signal molecules mandatory to guide maturing T cells, to support the T cell phenotype and to direct the differentiation in particular T cell subsets. Besides, cultured human primary mTECs conserved their propensity to express TSA molecules required to enable the tolerance to self-molecules and to sensor their environment. These findings are in line with the report of Nazzal et al. (11) that demonstrate that human primary mTECs can promote the proliferation of newly generated $CD25^+$ T cells from $CD4^+CD25^-$ T cells, protect Treg cells from cell death and preserve the phenotype and the function of Treg cells *in vitro*. As an interesting point of view, this model conserved the mTEC main features and functions. In the context of thymic related disease such as autoimmune myasthenia gravis, culturing mTECs is a good model to decipher the TEC physiopathological and functional disturbances underlining the chronic inflammation and the autoimmune reactions. Therefore, here we report a stable and robust model to culture human primary mTECs from the normal or pathological thymus. This model should yield valuable insight into the regulation of transduction pathway involved in thymic aging process as well as autoimmune pathological context.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of by the local French ethics committee. The protocol was approved by the by the local ethics committee (CPP, Kremlin-Bicêtre, France: agreement No. 06-018; CCP Ile de France Paris 7, France agreement No. C09-36). Subjects have received an informed consent for the use of thymic fragments.

AUTHOR CONTRIBUTIONS

ND and JV performed the experiments, analyzed the data, and interpreted the results. FT provided helps to obtain tissues. RR provided human thymic tissues. AG and RLP provided helpful suggestions to design experiments. SB-A was involved in all aspects of the study. ND wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01663/full#supplementary-material.

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Article #2

IL-23/Th17 cell pathway: A promising target to alleviate thymic inflammation maintenance in myasthenia gravis

Objective

The thymic chronic inflammation and activation of pathway-sustaining germinal center production have suggested an active role for thymic epithelial cells (TECs) in the pathological events occurring in the thymus of AChR$^+$ MG patients. Moreover, previous studies from the laboratory have also demonstrated 1) an activation of the interferon pathway in AChR$^+$ MG TECs, 2) dysfunctional signal delivery from AChR$^+$ MG TEC to T-cells during activation and maturation, and 3) over-secretion of chemokines by AChR$^+$ MG TECs.

To understand the contribution of mechanisms and pathways managed by TECs in AChR$^+$ MG thymic pathological and physiological events, we investigated the secretion of pro-inflammatory cytokines and their contribution to the inflammation process mediated by Th17 cells. The aim of this study was to analyze the molecular and cellular events mediated by TECs and their effects on inflammatory T-cells (Th17).

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Il-23/Th17 cell pathway: A promising target to alleviate thymic inflammation maintenance in myasthenia gravis

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ABSTRACT

IL-23/Th17 pathway has been identified to sustain inflammatory condition in several autoimmune diseases and therefore being targeted in various therapeutic and effective approaches. Patients affected with autoimmune myasthenia gravis exhibit a disease effector tissue, the thymus, that harbors ectopic germinal centers that sustain production of auto-antibodies, targeting proteins located in the neuromuscular junction, cause of the organ-specific chronic autoimmune disease.

The present study aims to investigate the IL-23/Th17 cell pathway in the thymic inflammatory and pathogenic events.

We found that thymuses of MG patients displayed overexpression of Interleukin-17, signature cytokine of activated Th17 cells. This activation was sustained by a higher secretion of Interleukin-23 by TEC, in addition to the increased expression of cytokines involved in Th17 cell development. The overexpression of Interleukin-23 was due to a dysregulation of interferon type I pathway. Besides, Interleukin-17 secreted, and Th17 cells were localized around thymic ectopic germinal centers. These cells expressed podoplanin, a protein involved in B-cell maturation and antibody secretion. Finally, production of Interleukin-23 was also promoted by Interleukin-17 secreted itself by Th17 cells, highlighting a chronic loop of inflammation sustained by thymic cell interaction.

Activation of the IL-23/Th17 pathway in the thymus of autoimmune myasthenia gravis patients creates an unstoppable loop of inflammation that may participate in ectopic germinal center maintenance. To alleviate the physio-pathological events in myasthenia gravis patients, this pathway may be considered as a new therapeutic target.

1. Introduction

Myasthenia gravis (MG) is an organ-specific chronic autoimmune disease caused by auto-antibodies that target proteins located in the neuromuscular junction. Most patients have antibodies directed against acetylcholine receptor (AChR). These patients commonly present thymic abnormalities such as follicular hyperplasia or thymoma in early and late onset patients, respectively [1]. Sudres et al. have demonstrated that MG thymuses contain the pathological factors, including antibodies and defective T cells, required to induce MG symptoms in an immunodeficient mouse model [2]. A randomized clinical trial showed that thymectomy allows an amelioration of MG patient symptoms. However, this procedure is still not a cure since it does not fit to all patients, and most patients need other long-term therapies [3].

In AChR$^+$ MG patients, inflammation occurs in the thymus and is sustained by interferon-related molecules. This inflammation is

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Abbreviations

AChR	acetylcholine receptor
AID	activation-induced cytidine deaminase
CD	cluster of differentiation
CCL	chemokine (C-C motif) ligand
CCR6	C chemokine receptor 6
CXCR5	C-X-C chemokine receptor type 5
DC	dendritic cell
EAMG	experimental autoimmune myasthenia gravis mouse model
eGC	ectopic Germinal center
FDA	food and drug administration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
MG	myasthenia gravis
mRNA	messenger ribonucleic acid
PBMC	peripheral blood cell
qPCR	quantitative polymerase chain reaction
PDPN	podoplanin
Poly(I:C)	polyinosinic-polycytidylic acid
RORγT	retinoic acid-related orphan receptor gamma T
TEC	thymic epithelial cell
TGF	tumor growth factor
Th	T-helper cell
TNFα	tumor necrosis factor α
Treg	regulatory T cell

described to be driven by the secretion of pro-inflammatory cytokines (Interleukin (IL)-6, Interferon γ (IFN-γ), tumor necrosis factor α (TNFα) and IL-17 [4,5]. An altered development and function of different subpopulations of T cells, such as regulatory T cells (Tregs) and T helper 17 cells (Th17), can be the result of the tissue microenvironment cytokines content. Accumulating data have illustrated that a disequilibrium between these two subpopulations of T cells is often involved in the pathogenesis of autoimmune diseases. In AChR$^+$ MG patients, there is a strong defective function of Treg cells [6,7]. In addition, cytokines related to Th17 cells (IL-17 and IL-21) are increased in Treg cells and also in conventional T cells in the thymus and peripheral blood cells (PBMCs) of MG patients compared with controls [8,9].

AChR$^+$ MG hyperplastic thymuses harbor ectopic germinal centers (eGCs), structures where B-cells are chemo-attracted and activated resulting in antibody production [10]. In various models of experimental autoimmune disease, IL-17 secreting CD4$^+$ T cells participate to the loss of B-cell tolerance [11,12] and probably to eGC antibody secretion process [13] by expressing an anchoring protein, podoplanin (PDPN) [14] to stabilize their effects within the eGCs [15]. Previous studies, by using mice with spontaneous experimental autoimmune encephalomyelitis, have shown a correlation between PDPN expression levels and the formation of eGCs in the central nervous system. By blocking PDPN, the formation of eGCs is stopped, and disease progression and symptoms are partially prevented [16].

Therefore, we investigated the cellular components and the actors that may sustain IL-17 over-expression and its role in the thymic eGCs formation. Here, we quantified and located the Th17 cells inside the MG thymus. We then deciphered the level of expression of cytokines involved in the Th17-cell differentiation and activation processes, and the cellular mechanisms underlining their over-activation in MG thymuses. We identified an uncontrolled loop, involving IL-23, thymic epithelial cells (TECs), Th17 cells, PDPN and type 1 interferon (IFN-I) pathway, that may sustain the chronic thymic inflammation process and probably participate in antibody production.

2. Material and methods

2.1. Thymic biopsies

Control and MG thymic biopsies were obtained from patients undergoing respectively corrective cardiovascular surgery or thymectomy at Marie Lannelongue Chirurgical Center (Le Plessis-Robinson, France) and at the Strasbourg civil hospital (Strasbourg, France). 51 healthy controls (aged of two days to 45 years old) were used. 72 MG thymuses (aged of 12–53 years old) were included in the study (Table 1).

2.2. Human serum and blood cells

Human serum and peripheral blood mononuclear cells (PBMCs) were obtained from whole blood of healthy patients collected by the Etablissement Français du Sang and AChR$^+$ MG patients during their clinical follow up.

Clinical data of MG patients are summarized in Table 1. The study was under the French Bioethic Law that requires a written informed consent from the donors or the legal representant. In respect to this law, this study was approved by the local ethics committee (CPP, Kremlin-Bicêtre, France: agreement N°06-018; CCP Ile de France Paris 7, France agreement N°C09-36).

2.3. Primary cell cultures

Primary human thymic epithelial cell (TEC) cultures were established following the protocol previously described [17,18]. After 7 days of primary culture, cells were trypsinized, seeded in RPMI medium containing 5% of horse serum and then allowed to attach to the flask for 24 h before treatment. Cells were treated in RPMI medium containing 0.5% of horse serum for 24 h with Poly (I:C) (100 μg/ml; InvivoGen, Toulouse, France), IL-17A (100 pg/ml or 10 ng/ml; R&D systems, Lille, France), anti-IL-17 receptor antibody (1 μg/ml, R&D systems, Lille, France), IFN-γ (1000UI/ml; R&D systems, Lille, France), LPS (10 ng/ml, Enzo Life Sciences, Villeurbanne, France), IFN-type I (1000UI; R&D systems, Lille, France) and anti-IFNα/β receptor (10 μg/ml; Enzo Life Sciences, Villeurbanne, France).

2.4. Co-culture assays

Freshly Ficoll isolated PBMCs were seeded into 12-well plates in an RPMI 1640 Glutamax I medium supplemented with 10% fetal calf serum, alone or with primary cultured TECs in a ratio of 2:1. A capture antibody anti-IL-23p19 (1 ng/ml; R&D systems, Lille, France) was added in the culture medium. TECs and PBMCs were incubated at 37 °C in contact for 24 h. For FACS analysis, to inhibit cytokine secretion by PBMCs, Brefeldin A (10 μg/ml; Sigma-Aldrich, Lyon, France) was added to the culture medium for 4 h before the cells staining. For RNA analysis, PBMCs and TECs were washed twice with PBS 1X and quick frozen prior RNA extractions.

2.5. Quantitative real-time PCR

Gene expression was evaluated by quantitative real-time PCR performed using the Light-Cycler apparatus (Roche Diagnostics; Meylan, France) as previously described by Dragin et al. [19]. Arbitrary units were calculated as previously described [4,20]. Each PCR was performed using the Fast-start DNA Master SYBR Green I kit (Roche Diagnostics; Meylan, France) according to the manufacturer's instruction. Each cDNA sample was run in duplicate. mRNA expression was normalized to 28S for thymic global biopsies or GAPDH for purified thymocytes, PBMCs or cultured cells. The list of primers used is

Table 1
Clinical data of Myasthenia Gravis patients. The degree of thymic hyperplasia was determined by an anatomo-pathologist that quantified the number of germinal centers by thymic sections as followed: ++++:very numerous; +++: numerous; ++:Some, +:Rare; –:No clear germinal centers. NA: not available; NT: No treatment; IVIG: Intravenous Immunoglobulins.

	GENDER	AGE OF THYMECTOMY	DEGREE OF HYPERPLASIA	CHOLINESTERASE TREATMENT	CORTICOIDS & OTHER TREATMENTS	TECs	THYMOCYTES	THYMUS	SERUM
MG 1	F	14	++	NA	NA	X			
MG 2	F	27	++	mestinon	NT	X			
MG 3	F	28	–	mestinon	IVIG	X			
MG 4	F	37	++	mestinon	NT	X			
MG 5	F	19	+++	Yes	NT	X			
MG 6	F	40	+++	mestinon	NT	X			
MG 7	F	27	++	mestinon	NT	X			
MG 8	F	19	–	mestinon	NT	X	X		
MG 9	F	18	+++	mytelase	NT	X			
MG 10	M	21	–	mestinon	NT	X			
MG 11	F	32	+	mytelase	Mycophenolate mofetil	X			
MG 12	M	12	++	mytelase	IVIG	X			
MG 13	F	30	–	mestinon	IVIG	X			
MG 14	F	22	++	mytelase	IVIG	X			
MG 15	M	13	+++	NT	NT	X			
MG 16	M	41	+++	mytelase	NT	X			
MG 17	F	18	–	mestinon	Corticoids/IVIG	X			
MG 18	F	12	+++	mytelase	NT	X			
MG 19	F	14	++	mestinon	NT	X			
MG 20	F	38	++	mestinon	NT	X			
MG 21	F	29	++	mestinon	NT	X			
MG 22	F	36	+++	mestinon	NT		X		
MG 23	F	29	+	mestinon	Prednisone/IVIG		X		
MG 24	F	26	+++	mestinon	NT		X		
MG 25	F	25	+++	mytelase	NT		X		
MG 26	F	22	++++	mytelase	NT		X		
MG 27	F	22	++++	NA	NA		X		
MG 28	M	31	NA	NA	NA		X		
MG 29	F	29	++	mestinon	NT		X		
MG 30	M	19	+++	mestinon	NT		X		
MG 31	F	20	+++	mestinon	NT		X		
MG 32	F	24	++++	mytelase	NT		X		
MG 33	F	25	+++	mytelase	NT		X		
MG 34	M	25	++	mestinon	NT		X		
MG 35	F	16	++	mytelase	NT		X		
MG 36	F	16	++++	mytelase	NT		X		
MG 37	F	41	+++	mestinon	NT		X		
MG 38	F	32	++++	mytelase	NT		X		
MG 39	F	23	++	mestinon	NT		X		
MG 40	F	17	++	mestinon	NT		X		
MG 41	F	26	++	mestinon	NT		X		
MG 42	M	30	–	mytelase	NT		X		
MG 43	M	29	++	mytelase	NT		X		
MG 44	M	27	++	mytelase	NT		X		
MG 45	F	22	+++	mestinon	NT		X		
MG 46	F	22	+++	mestinon	NT		X		
MG 47	F	18	+++	mytelase	NT		X		
MG 48	M	53	++	mytelase	NT			X	
MG 49	F	44	++	mestinon	NT			X	
MG 50	M	38	–	mytelase	NT			X	
MG 51	F	16	–	mytelase	NT			X	
MG 52	F	50	++	mytelase	NT			X	
MG 53	F	18	++++	mestinon/mytelase	NT			X	
MG 54	F	14	+++	mestinon	IVIG			X	
MG 55	F	32	++	mestinon	NT			X	
MG 56	F	26	++	mytelase	NT			X	
MG 57	F	20	++	mestinon	NT			X	
MG 58	F	21	++++	mytelase	NT			X	
MG 59	F	34	++	mestinon	NT			X	
MG 60	F	15	++	mestinon	cortancyl			X	
MG 61	F	22	–	mestinon	cortancyl/IVIG			X	
MG 62	F	17	–	mytelase	NT			X	
MG 63	F	44	–	mestinon	cortancyl			X	
MG 64	F	32	++	mytelase	NT			X	
MG 65	F	33	–	mestinon/mytelase	cortancyl			X	
MG 66	F	35	–	NT	NT			X	
MG 67	F	24	+++	mestinon/mytelase	NT			X	
MG 68	F	35	+++	mestinon/mytelase	NT			X	
MG 69	F	29	+++	mytelase	NT			X	
MG 70	F	24	+++	mytelase	NT			X	
MG 71	F	25	+++	mytelase	NT			X	

(*continued on next page*)

Table 1 (*continued*)

	GENDER	AGE OF THYMECTOMY	DEGREE OF HYPERPLASIA	CHOLINESTERASE TREATMENT	CORTICOIDS & OTHER TREATMENTS	TECs	THYMOCYTES	THYMUS	SERUM
MG 72	F	33	–	mestinon/mytelase	cortancyl			X	
MG 73	F	38	+ + +	mytelase	NT				X
MG 74	F	19	+ + +	mestinon	NT				X
MG 75	F	32	+ +	mytelase	NT				X
MG 76	F	14	+ +	mytelase	NT				X
MG 77	F	40	+ + +	mytelase	NT				X
MG 78	F	29	+ + +	mestinon	NT				X
MG 79	F	31	+ +	mestinon	NT				X
MG 80	F	19	+ + +	mestinon	NT				X
MG 81	F	19	–	NT	cortancyl				X
MG 82	M	21	–	NT	cortancyl				X
MG 83	F	23	+ + +	mestinon	solupred				X
MG 84	F	26	+	imurel/mestinon	cortancyl				X
MG 85	M	28	–	mestinon	cortancyl				X
MG 86	F	29	–	mestinon	cortancyl				X
MG 87	M	31	+ + + +	mestinon	cortancyl				X
MG 88	M	35	–	mytelase	cortancyl				X
MG 89	F	35	–	imurel/mestinon	cortancyl				X
MG 90	M	38	+ + + +	mestinon	corticoids				X
MG 91	M	19	+ + + +	mestinon	NT				X
MG 92	F	24	+ + +	mytelase	NT				X
MG 93	M	25	+ +	mestinon	NT				X
MG 94	F	26	+ + + +	mestinon	NT				X
MG 95	F	29	+ + + +	mytelase	NT				X
MG 96	M	29	+ + + +	mestinon	NT				X
MG 97	F	35	+ +	mestinon	NT				X
MG 98	F	36	+ +	mestinon	NT				X
MG 99	F	39	+ + +	mytelase	NT				X
MG 100	M	27	+ +	mytelase	NT				X

summarized in Table 2.

2.6. ELISA

The levels of cytokines (IL-17, IL-23, IL-6, IL-1β, TGFβ3) were analyzed in serum and TEC supernatants using the DuoSet Elisa kit (R& D systems, Lille, France). For total thymic protein analysis, we followed the protocol previously described by Meraouna et al. [10]. Total thymic proteins were extracted in solution containing 5% Tris HCl 20 mM, 0.1% Triton X100, and one tablet of protease inhibitor cocktail (complete-mini; Roche-Diagnostics, Meylan, France) using the fast prep apparatus. Each ELISA was performed according to the manufacturer's instructions. ELISA reactions were read with a TECAN SPARK ELISA microplate reader.

2.7. Flow cytometry analyses

To analyze the secretion of IL-17 by thymocytes, frozen MG and control cells were unfrozen in fetal bovine serum. Cells were incubated in X-vivo medium and stimulated with Phorbol 12-Myristate 13-Acetate (100 ng/ml, Sigma-Aldrich, Lyon France), Ionomycin (1 μg/ml; Sigma-Aldrich, Lyon, France) and Brefeldin A (10 μg/ml; Sigma-Aldrich, Lyon, France) for 4 h. Activated thymocytes were then stained with fluorochrome-conjugated antibodies for 30 min at 4 °C before being permeabilized with the FoxP3 permeabilization kit (eBioscience, Paris, France) and labeled with anti-IL-17A antibody according to the manufacturer's instruction. All analyses were done with the cytometer FACS Canto II (BD Biosciences, Le Pont de Claix, France).

The same procedure was used for PBMCs recovered from co-cultured experiments.

2.8. Immunofluorescence microscopy

Cryostat sections (7 μm) of frozen human thymic tissues were fixed with acetone to glass superfrost slides and dried for 1 h. The human thymic sections were pre-incubated with a blocking buffer (PBS, 0.1% BSA, 10% FBS, 0.3 M Glycine, 1%Tween) for 1 h at room temperature and then, incubated overnight at 4 °C with antibodies raised against human antigens. The labeled cells were revealed with Alexa 488, Alexa 594, Alexa 350 and or Alexa 647 coupled secondary IgG raised in donkey, chicken or rat. Labelling were performed as previously described [18]. Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope using 20× magnification (Carl Zeiss, Le Pecq, France).

2.9. Statistical analysis

Non-parametric tests (Wilcoxon test for paired data or Mann-Whitney test for unpaired values) were used to compare groups as specified in each Fig. legend. Values were reported as Mean ± Sem. *GraphPad Prism 5* software was used to generate the graphs and to perform the statistical analysis. Statistical significance was recognized at $p < 0.05$.

Table 2
List of primers used in the study.

Gene	Primer #1	Primer #2
28S	GGTAGGGACAGTGGGAATCT	CGGGTAAACGGCGGGAGTAA
AID	AAGGGCTGCATGAAAATTCAGT	CGTCTCGTAAGTCATCAACCTC
CD4	CCTGGTAGTAGCCCCTCAGT	CTGGAAAGCTAGCACCACGA
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG
IFN-γ	TCCCATGGGTTGTGTGTTTA	AAGCACCAGGCATGAAATCT
IL-1β	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA
IL-6	TGAGGTGCCCATGCTACATTT	TCTGCGCAGCTTTAAGGAGTT
IL-17A	CCCCTAGACTCAGGCTTCCT	AGTTCATTCTGCCCCATCAG
IL-17R	CACTAGCCTTTTGGGCTCAG	TACGCAGGAAGAGTGCATTG
IL-21	GGCAACATGGAGAGGATTGT	AAGCAGGAAAAAGCTGACCA
IL-23	CAGCAACCCTGAGTCCCTAA	CCAAATTTCCCTTCCCATCT
IL-23R	TGCCTTGCAATCTGAACTTG	GAGCTCCCGGGAATTCTTAC
Podoplanin	TGTGGCGCTTGGACTTTGT	GTGTAACAGGCATTCGCATCG
RORC	CAAGAGAGGTTCTGGGCAAG	AGTGGGAAGGCAAGATCAGA
STAT3	CTGGCCTTTGGTGTTGAAAT	AAGGCACCCACAGAAACAAC
TGF-β3	AACGGTGATGACCCACGTC	CCGACTCGGTGTTTTCCTGG

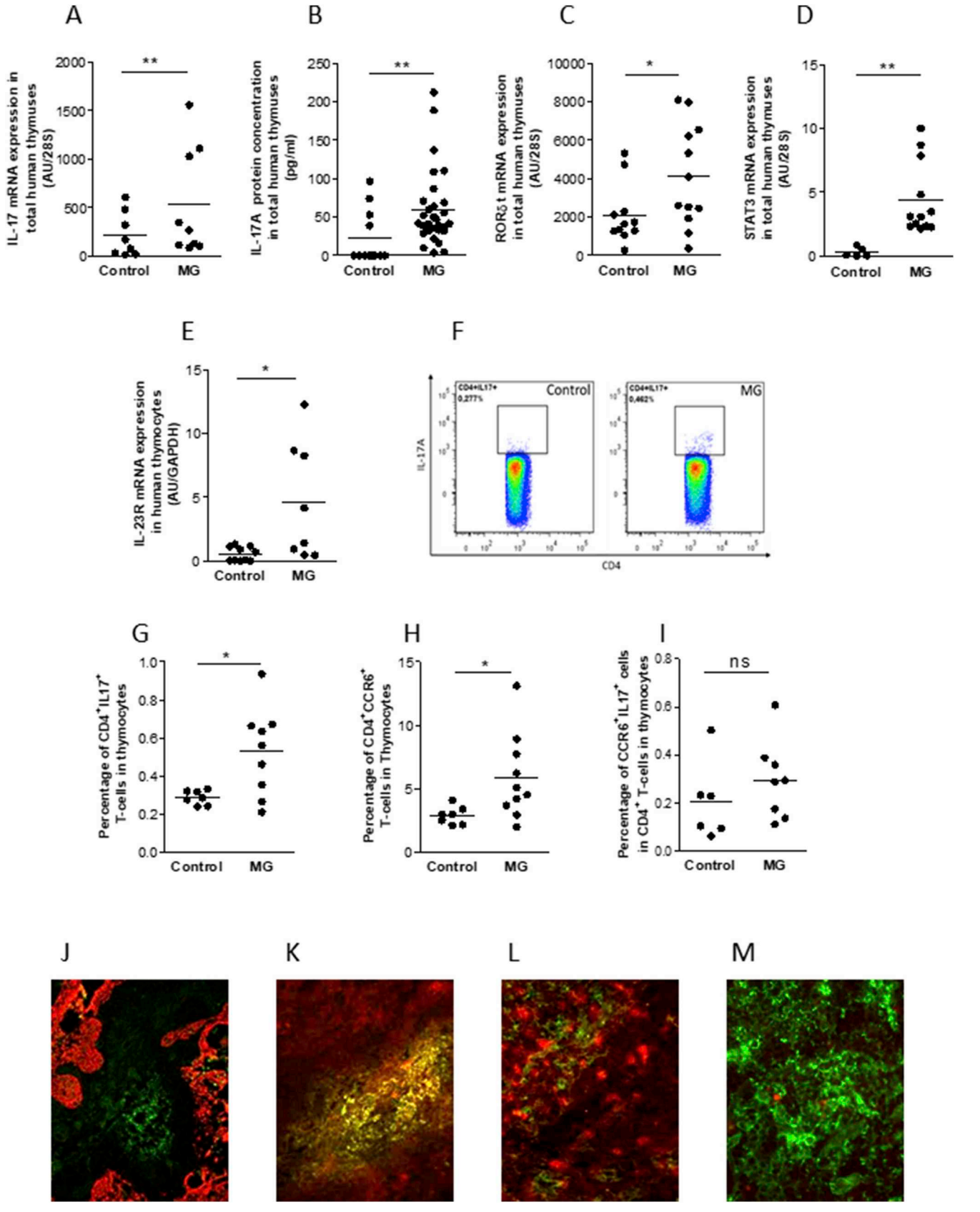

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Fig. 1. Expression of IL-17 in thymuses of AChR$^+$ MG patients. Compared analyses in global thymic biopsies obtained from AChR$^+$ MG patients and aged-matched controls of IL-17 expression **A)** at the mRNA and **B)** protein levels, **C)** of RORγt and **D)** STAT3 at the mRNA expression level. **E)** mRNA analysis of IL-23 receptor in purified human thymocytes from MG thymuses compared to aged matched controls. **F)** Representative image of Flow cytometry analysis of CD4^{sp}IL-17$^+$ cells in the thymocytes from AChR$^+$ MG patients and aged matched controls. **G)** Analysis by flow cytometry of the percentage of CD4^{sp} cells expressing IL-17 in purified human thymocytes from AChR$^+$ MG patients and aged matched controls **H)** Analysis by flow cytometry of the percentage of CD4$^+$CCR6$^+$ cells in human purified thymocytes from AChR$^+$ MG patients and aged matched controls. **I)** Analysis by flow cytometry of the percentage of CCR6$^+$IL17$^+$ cells in CD4^{sp} cells in purified human thymocytes from AChR$^+$ MG patients and aged matched controls. Representative images of AChR$^+$ MG human thymic sections co-labeled with antibodies anti-IL-17 (green) and anti keratin 5/14 (red) **J)** or anti CD21 (red) **K)** or anti CD11c (red) **L)** or anti FoxP3 (red) **M).** mRNA expression was analyzed by real-time PCR and is expressed as arbitrary unit (AU) normalized to 28S or GAPDH. Proteins were analyzed by ELISA. Each point represents the mean value of a duplicate analysis of each donor. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope. N > 5 for control thymuses and n > 9 for AChR$^+$ MG thymuses for mRNA and protein analyses. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*p < 0.05; **p < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. IL-17$^+$ cells in the thymus of AChR$^+$ MG patients

Th17 cells and their signature cytokine, IL-17, are involved in pro-inflammatory mechanisms that sustain the development of autoimmune diseases. Gradolatto et al., by using microarray analysis, have reported the over-expression of IL-17, at the mRNA level, in purified thymocytes of AChR$^+$ MG patients in both Treg and conventional CD4$^+$ T cells [8].

Here, we validated this observation and demonstrated that the expression level of IL-17 in AChR$^+$ MG thymus was increased at the mRNA level (Fig. 1A) as well as at the protein level (Fig. 1B). To prove that this IL-17 signature was linked to Th17 cells, we analyzed the mRNA expression of RORγT (the master transcription factor of Th17 cells), STAT3 (required for Th17 cell differentiation) and IL-23 receptor (IL-23R), a membrane protein classically expressed by Th17 cells. We observed a significant overexpression of RORγt (Fig. 1C), STAT3 (Fig. 1D) and of IL-23R (Fig. 1E) in AChR$^+$ MG samples at the mRNA level. To further characterize the IL-17 producing cells in thymi of AChR$^+$ MG patients, we analyzed by flow cytometry T cell sub-types using specific surface markers (CD4, CD8, IL23R, CCR6) in purified thymic cells from control and MG patients. We observed a higher percentage of CD4$^+$ (CD4$^+$CD8$^-$) cells expressing IL-17 in AChR$^+$ MG thymuses compared to controls (Fig. 1F and G). CCR6 is a chemokine receptor associated with Th17 cell phenotype. We observed a significant increased percentage of CD4$^+$CCR6$^+$ T cells (Fig. 1H). The percentage of CCR6$^+$IL-17$^+$ T cells (Fig. 1I), was also increased although not significant.

To understand the potential role of the IL-17 expressing cells, inside MG thymuses, we performed immunohistochemistry analyses of MG and control thymuses. Our analysis showed the presence of IL-17 producing cells within eGCs (Fig. 1J and K), suggesting an involvement of IL-17 in the eGC homeostasis. In addition, we co-stained AChR$^+$ MG thymic sections with IL-17 and CD11c (marker for dendritic cells) (Fig. 1L) or FoxP3 (Treg cell marker) (Fig. 1M) and did not observe a co-localization, minimizing the contribution of these cells in the global production of IL-17. These data corroborate findings observed in other autoimmune diseases [21] suggesting an involvement of IL-17 producing cells in B-cell activation and stabilization inside the eGCs. Thus, in regards to what has already been shown in various autoimmune diseases, it is possible that in AChR$^+$ MG thymus, IL-17 is involved in the formation of eGCs.

3.2. T cells expressing podoplanin are contained in MG thymic eGCs

Effector T cells and mainly Th17 cells that actively contribute to eGCs formation and homeostasis, express PDPN during autoimmune inflammation [15]. Therefore, we wondered whether PDPN was expressed in T cells surrounding the eGCs of AChR$^+$ MG thymus. We first investigated the mRNA expression of PDPN in control and MG AChR$^+$ thymuses. mRNA analysis showed a significant increase in PDPN in total thymus extracts of MG patients (Fig. 2A). This increase was due to the lymphocyte population (Fig. 2B) and not to the TECs that displayed a decreased expression in PDPN in MG thymuses (Fig. 2C). We then corroborated the expression of PDPN by flow cytometry analysis of purified thymic lymphocytes. We observed a significant increase in CD4$^+$ cells expressing PDPN (Fig. 2D) and CD4$^+$IL23R$^+$ PDPN$^+$ cells subset (Th17 cells) (Fig. 2E and F). By performing IHC analyses, we observed that PDPN was mostly present in the interlobular zones in control thymic sections (Fig. 2G) while PDPN co-localized with CD4 positive, IL-23R positive cells found in MG thymic eGCs (Fig. 2H and J). eGCs are complex structures where both Th17 and Tfh cells collaborate to the development of B cells [22]. In order to clarify whether CD4$^+$ PDPN$^+$ cells or IL23R$^+$ PDPN$^+$ cells present in thymic MG eGCs, were not Tfh cells or B cells, we co-stained thymic MG sections with PDPN, CD20 and CXCR5 antibodies. Fig. 2K and L showed that cells expressing PDPN are not CXCR5$^+$ cells (Tfh cells) neither CD20$^+$ cells suggesting that cells expressing podoplanin in MG thymic eGCs are most likely to be Th17 cells. In addition, in the thymic biopsies of AChR$^+$ MG patients, we observed an increased expression of activation-induced cytidine deaminase (AID) (Fig. 2M), a protein highly expressed in eGCs that contributes to B cells activation and clonal expansion, emphasizing the key role of Th17/PDPN cells in the GC formation and maintenance.

3.3. Th17 cell differentiation and activation in thymuses of AChR$^+$ MG patients

Differentiation of naïve CD4$^+$ T cells into Th17/IL-17 secreting cells is a complex process highly dependent on the presence of different cytokines including the transforming growth factor-β1 and 3 (TGFβ-1, TGFβ-3), IL-6, IL-1β, IL-21 and IL-23 [22,23]. In this context, we evaluated the thymic expression of these cytokines in AChR$^+$ MG patients. The mRNA expression levels of IL-6, TGF-β3, IL-1 β, IL-21 and IL-23 were significantly increased in AChR$^+$ MG thymus compared to age-matched controls (Fig. 3A–E). In parallel, in the sera, we observed that among all these cytokines, the protein level of only IL-23 was significantly over-expressed in AChR$^+$ MG patients (Fig. 3F and J). Since IL-23 is responsible for Th17-cell activation towards a pathogenic phenotype and also for stimulating IL-17 expression, these data suggest that in AChR$^+$ MG thymuses an active process may sustain the CD4$^+$ T cell differentiation into active and pathogenic Th17 cells through IL-23 pathway that is perpetuated in the periphery.

3.4. TECs are main IL-23 producer cells in MG thymuses

To identify and to decipher the mechanism underlining the overproduction of IL-23, we evaluated the IL-23 expression level in the two main thymic cell subtypes (thymic lymphocytes and TECs). We found no significant difference of IL-23 expression in AChR$^+$ MG thymic lymphocytes compared to control ones (Fig. 4A). However, TECs from AChR$^+$ MG patients displayed an mRNA overexpression of IL-23 as compared to controls (Fig. 4B). In addition, protein analysis of TEC supernatants corroborated the overproduction of IL-23 by MG TECs (Fig. 4C).

In order to validate the overexpression of IL-23 by MG TECs, and since IL-23 is known to be expressed by activated dendritic cells (DCs)

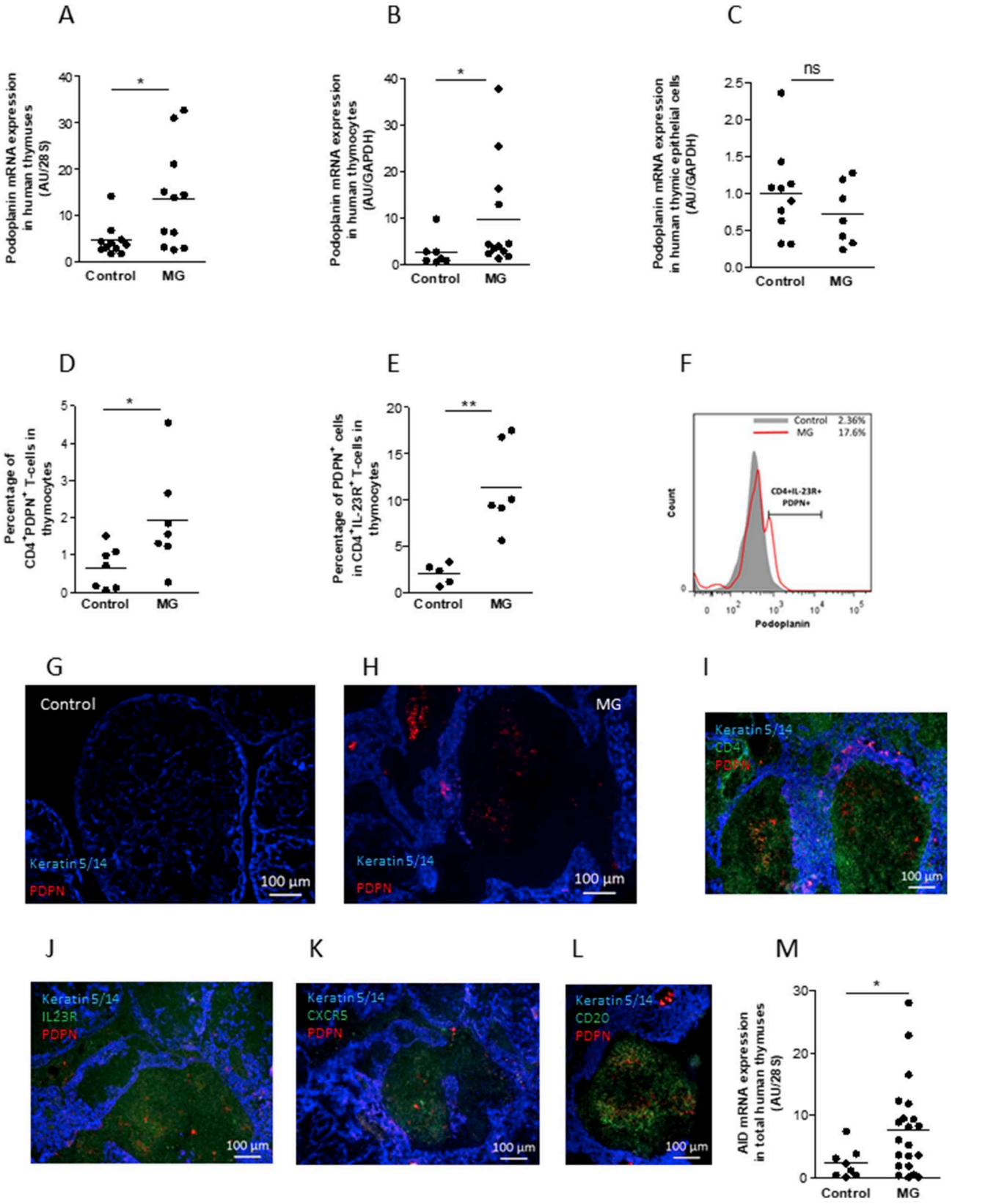

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Fig. 2. Podoplanin expression in AChR$^+$ MG thymuses. Comparative analysis of podoplanin mRNA expression in **A)** global thymic biopsies, **B)** purified human thymocytes and **C)** primary cultured thymic epithelial cells obtained from AChR$^+$ MG patients and controls individuals. Analysis by flow cytometry of the percentage **D)** of CD4$^+$PDPN$^+$ cells, **E)** of CD4$^+$IL23R$^+$ cells expressing Podoplanin in the purified thymocytes obtained from AChR$^+$ MG patients and controls individuals. **F)** Representative graph of flow cytometry analysis of PDPN expression in CD4$^+$IL-23R$^+$ cells in purified thymocytes from AChR$^+$ MG patients and controls. Representative thymic sections of control **G)** and AChR$^+$ MG patient **H)** co labeled with antibodies anti Podoplanin (red), and anti Keratin 5/14 (blue). Thymic sections of AChR$^+$ MG patient co-labeled with antibody anti Keratin 5/14 (blue), Podoplanin (red) and anti CD4 (green) **I)** or anti IL23R (green) **J)** or anti CXCR5 (green) **K)** or anti CD20 (green) **L)**. Comparative analysis of AID mRNA expression in global thymic biopsies from AChR$^+$ MG patients and controls individuals **M)**. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope using 20× magnification. For global thymus analyses, N > 4 for control and n > 4 for AChR$^+$ MG thymuses. mRNA expression was analyzed by real-time PCR and is expressed as arbitrary unit (AU) normalized to 28S or GAPDH. Each point represents the mean value of duplicate analysis of each donor. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and phagocytic cells [24], we proceeded with in situ protein identification. As observed in Fig. 4D, immunohistochemical analysis of control thymus shows a subtle and focused IL-23 protein staining mainly in the Hassall's corpuscles while AChR$^+$ MG thymuses display IL-23 staining in the interlobular zone, the germinal centers (Fig. 4E–H) as well as in keratin 5/14 positive cells (Fig. 4H). More, in agreement with previous studies reporting dendritic cell propensity to express IL-23 [24], we observed few dendritic cells (CD11c$^+$) expressing IL-23 in AChR$^+$ MG thymic biopsies (Fig. 4I), even-though their expression remained negligible as compared to the TEC levels. Altogether, these results demonstrated that in AChR$^+$ MG thymuses, TECs were the main cells over-expressing IL-23, the Th17-cell activator. These data raise the question of the mechanism(s) underlining the over-expression of IL-23 in MG TECs.

3.5. Interferon type I pathway stimulates IL-23 expression in MG thymuses

We mimicked, in vitro, an inflamed environment by treating human control TECs with lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly (I:C)), a synthetic analog of double-stranded RNA to mimic viral infection). We observed that in control primary human TECs, after 24 h, LPS inhibited significantly IL-23 expression (Fig. 4J). While poly (I:C) stimulated it (Fig. 4J). Since Poly (I:C) activates the IFN-I pathway in human TECs [25], a pretreatment of human control TECs with an anti IFN α/β receptor blocking antibody (α-IFNαβ receptor) inhibited Poly (I:C) effect (Fig. 4J), confirming that poly (I:C) activated IL-23 expression through IFN-I transduction pathway.

To precise this assumption, human control TECs were challenged with the two types of interferon (IFN-I and IFN-γ). Fig. 4K shows that IL-23 expression is induced by IFN-I pathway activation and not by IFN-γ. In addition, AChR$^+$ MG TECs (cells that used to be in an inflammatory environment) remained responsive to Poly (I:C) (Fig. 4L) but displayed a lower ability to over-express IL-23 compared to controls, when stimulated with Poly (I:C), 2,6 Vs 5 fold change respectively (Fig. 4J and L). Fig. 4M illustrates the ratio of IL-23 expression in control versus MG TECs at steady state (Fig. 4B) and after Poly IC stimulation (Fig. 4J and L). Therefore, Fig. 4M demonstrates that whether MG TECs can overproduce IL-23, compared to control TECs, they are less inducible probably due to a constant in vivo stimulation that increased their steady state level of IL-23 production and consequently limit their capacity to be re-activated in vitro.

Altogether, these results demonstrate TECs participate in the amplification of the inflammation process in AChR$^+$ MG thymus through their over-production of IL-23.

3.6. MG TEC secretion of IL-23 increases IL-17 production

Considering that MG TECs over-express IL-23, IL-1β and IL-6 (cytokines involved in Th17 cell development), we suspected that crosstalk between TECs and T cells was important in the thymic inflammatory process in AChR$^+$ MG patients.

To validate our hypothesis and as a proof of concept, we performed co-cultures of primary control or MG TECs with fresh purified PBMCs from healthy patients. We used PBMCs to analyze TECs effects on CD4$^+$ mature cells and to avoid any confounding effects related to T cell maturation or developing process.

First, CD4 and IL23R expression levels were analyzed in PBMCs after co-culture. Results indicated that independently of the origin of the TECs (MG or control thymuses), when in contact with TECs, PBMCs harbored a decreased expression of CD4 and IL-23R, surface molecules (Fig. 5A–C), and no difference in CD8 (Fig. 5C). The decrease in IL-23R expression reinforces the anti-inflammatory effect of TECs described by Nazzal et al. [17]. More, previous reports have shown that a down-regulation of CD4 that occurs upon in vitro stimulation may lead to double negative T cells that exhibit an effector phenotype associated with an increased TCR dependent proliferation and increased production of IFN-γ and IL-17 [26].

Second the expressions of IL-17 and IFN-γ, cytokines expressed by Th17 and Th1 cells [27] respectively were assessed. When PBMCs were co-cultured with control TECs, no change was observed in IL-17 mRNA expression levels in PBMCs while a decrease in IFN-γ mRNA expression was observed (Fig. 5D and E). By contrast, PBMCs co-cultured with MG TECs displayed an increased mRNA expression of IL-17 but no change for IFN-γ (Fig. 5D and E). To determine the impact of IL-23 produced by MG TECs, we added a capture antibody anti-IL-23 in PBMCs co-cultured with MG TECs. The significant increase in the percentage of Th17 cells (CCR6$^+$IL17$^+$) within CD4$^+$ cells (Fig. 5F and G) is reduced although not significantly, by the anti-IL-23 (Fig. 5G). These results show that MG TECs production of IL-23 is partially involved in the differentiation of T cells into IL-17$^+$ T cells.

More, only PBMCs co-cultured with MG TECs over-expressed PDPN (Fig. 5H). The expression of PDPN in Th17 cells is known to be influenced by pro-inflammatory cytokines like IL-6 and IL-1β [28], both cytokines being upregulated in MG TECs [29]. Altogether our results suggest that MG TECs stimulate differentiation and activation of T cells into a Th17 cell phenotype expressing podoplanin.

3.7. T cell secretion of IL-17 promotes a retro activation of TECs

Our results suggest that MG thymuses display an inflammatory chronicity and protective bounders are ineffective to decrease/stop the “snowball” process. Therefore, we wondered whether a T cell retro-control on TECs may be also involved in this uncontrolled inflammation.

First, we observed no change in mRNA expression of IL-23 by control TECs in the presence of PBMCs (Fig. 6A) while MG TECs displayed an increased mRNA expression of IL-23 (Fig. 6A). To ensure the specificity of this effect, we analyzed TECs expression of IL-6, a cytokine known to be overexpressed by MG TECs. Similarly to IL-23, in co-culture with PBMCs, we did not observe any change in IL-6 mRNA expression by control TECs (Fig. 6B) while MG TECs displayed a significant over-expression of IL-6 (Fig. 6B).

Therefore, we hypothesized that PBMCs might produce factors that promote IL-23 production by MGs TECs among them IL-17. Hence, we treated control TECs with IL-17 at two different concentration levels found in AChR$^+$ MG thymus (0.1 ng/ml and 10 ng/ml) (Fig. 1B). mRNA expression of IL-23 was only modified by the higher IL-17 concentration (10 ng/ml) (Fig. 6C), an effect blocked by adding an antibody against

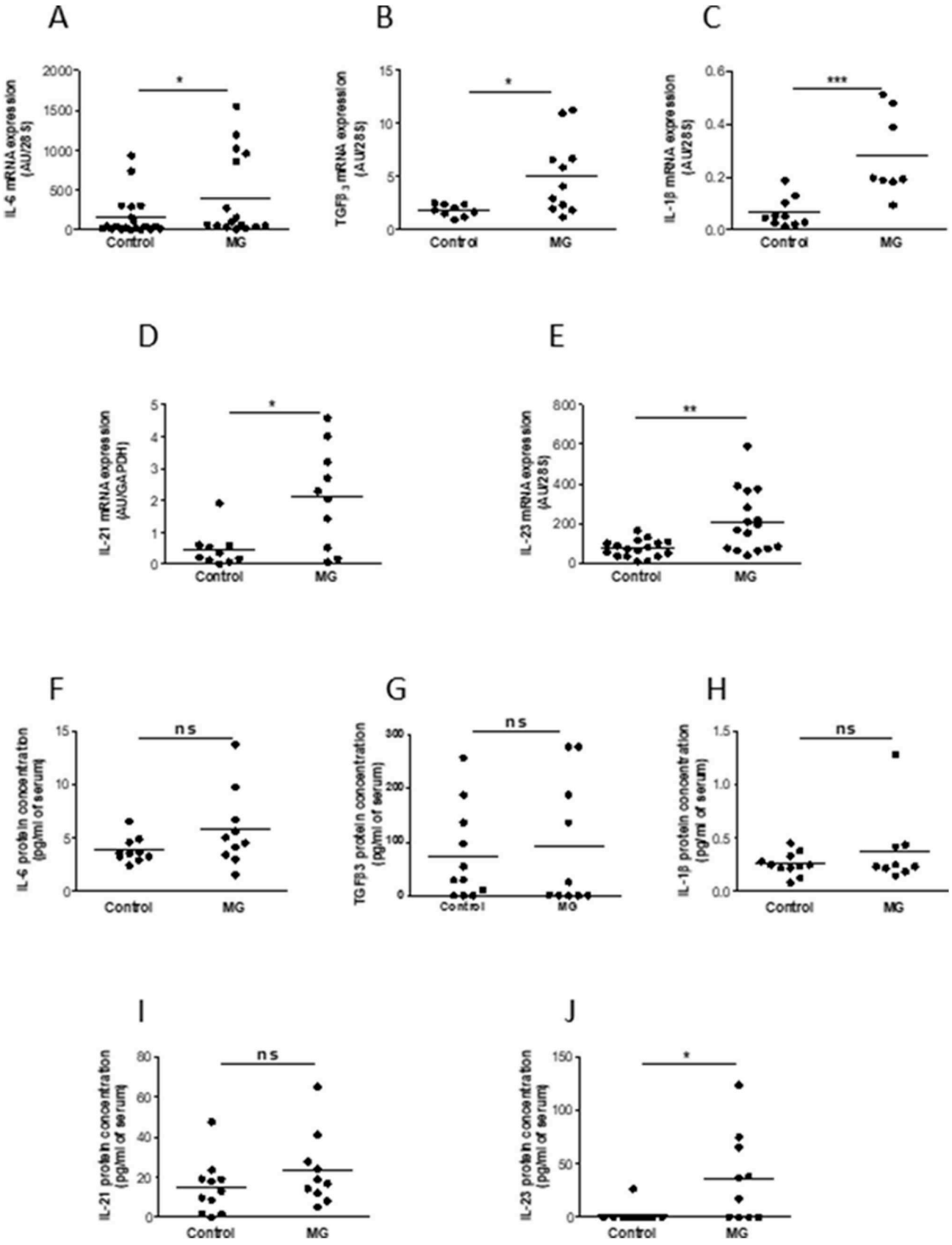

Fig. 3. Expression of cytokines involved in Th17 cells differentiation in thymuses of AChR$^+$ MG patients. mRNA expression levels of **A)** IL-6, **B)** TGF-β3, **C)** IL-1β, **D)** IL-21, and **E)** IL-23 in thymuses of AChR$^+$ MG patients compared to aged matched control adults. Protein expression level of **F)** IL-6, **G)** TGF-β3, **H)** IL-1β, **I)** IL-21 and **J)** IL-23 in sera of AChR$^+$ MG patients compared to aged matched controls. mRNA expression was analyzed by real-time PCR and normalized to 28S or GAPDH. mRNA are expressed as arbitrary unit (AU). Proteins were analyzed by ELISA. Each point represents the mean value of duplicate analysis of each donor. N $>$ 9 for control thymuses and n $>$ 8 for AChR$^+$ MG thymuses. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*p $<$ 0.05; **p $<$ 0.01; ***p $<$ 0.0005).

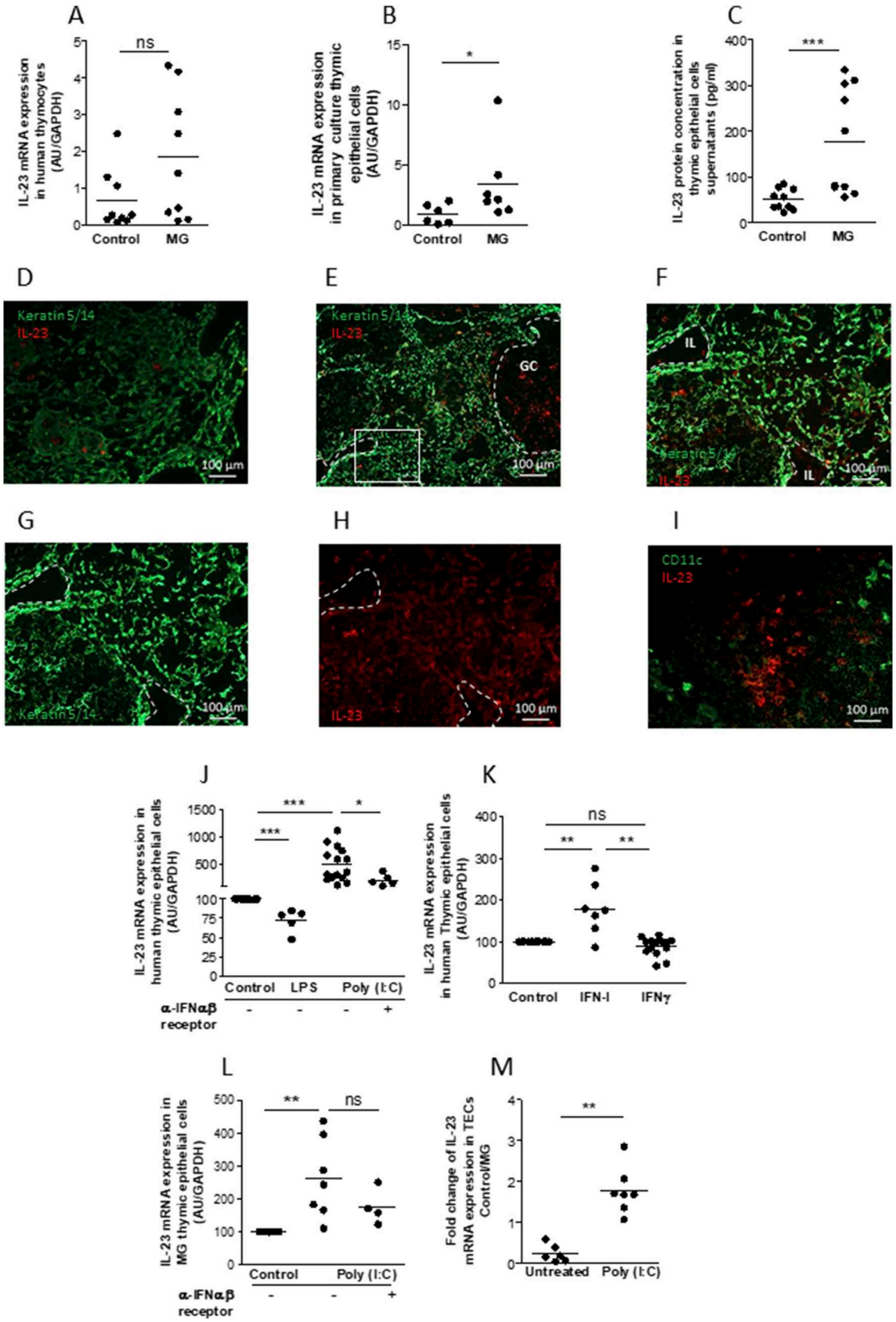

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Fig. 4. Overexpression of IL-23 is due to "inflamed" TECs in thymuses of MG patients. Analysis of mRNA expression level of IL-23 in **A)** thymocytes and **B)** primary cultured TECs of AChR$^+$ MG patients compared to controls. **C)** Protein expression level of IL-23 in supernatants of primary cultured TECs obtained from controls and MG patients. Representative picture of human thymic sections **D)** from controls and **E)** from AChR$^+$ MG patients co-labeled with an anti-IL-23 antibody (red) and antibody anti-keratin 5/14 (green) with a germinal center (GC). **(F–H)** Zoom of representative thymic human section of AChR$^+$ MG patient co-labeled with anti-IL-23 antibody (red) and antibody anti-keratin 5/14 (green) with interlobular area (IL). **I)** Representative pictures of AChR$^+$ MG patient thymic section co-labeled with an anti-IL-23 antibody (red) and an antibody anti CD11c (green). **J)** Effect of LPS (10 ng/ml) and Poly (I:C) (100 μg/ml) with or without an anti IFNα/β receptor (10 μg/ml), **K)** of type I IFN (1000UI/ml) or IFN-γ (1000UI/ml) on mRNA expression of IL-23 in human control TECs. **L)** Effect of Poly (I:C) (100 μg/ml) with or without an anti IFNα/β receptor (10 μg/ml) on IL-23 mRNA expression in AChR$^+$ MG TECs. **M)** Relative increased of IL-23 mRNA expression induced by Poly (I:C) in primary control TECs compared to AChR$^+$ MG. mRNA expression was analyzed by real-time PCR and normalized to GAPDH or Keratin 14. mRNA expression is expressed as arbitrary unit (AU). Proteins were analyzed by ELISA. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope. Each IHC labelling was done and repeated on thymic biopsies of different individuals. For global thymus analyses $n > 5$ for controls and $n > 9$ for MG thymuses. For analyses in primary cultured human TECs, cells were obtained from $n > 5$ different donors. Each point represents the mean value of duplicate analysis of each donor. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.0005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IL-17 receptor (α-IL-17R) (Fig. 6C). These data suggest that IL-23 over-expression by MG TECs could be reinforced by an IL-17 retro-control.

More, under IFN-I pathway activation through Poly (I:C), control TECs were able to produce IL-17 (Fig. 6D). Altogether, these data show that MG TECs chronic expression of IL-23 may be sustained by IL-17, a cytokine classically expressed by Th17 cells but also expressed by TECs following an IFN-I pathway activation. Therefore, an accumulation of IL-17 initiated by Th17 cells may help to develop a paracrine stimulation in TECs and possibly generating an unstoppable chronic inflammation (Fig. 6E).

4. Discussion

In autoimmune MG early-onset pathology, the thymus is an inflamed tissue that contains high levels of pro-inflammatory cytokines (IL-6, IFNγ, TGF-β, IL-1β) and chemokines (CXCL13, CCL21) that support the attraction of B-cells and the development of eGCs where production of pathogenic antibodies takes place.

We and others have previously analyzed the protein expression of different cytokines such as IL-6 [30–32], IL-1β [33] and TGF-β [34], and observed a higher expression of these cytokines in AChR$^+$ MG patients as compared with controls thymuses.

Here, we showed, for the first time, that in hyperplastic AChR$^+$ MG thymuses, the IL-23 pathway is implicated in a continuous loop of inflammatory events that sustains the development of Th17 cells and that may lead to eGC formation and probably sustain pathogenic antibody production.

4.1. MG TECs support thymic inflammation through Th17 cell activation

Th17 Cytokines are important controllers of the development of T cells. For instance, Th17 cell differentiation and activation require IL-6, IL-1β, IL-21, TGF-β1/3 and IL-23 [35]. IL-6 and TGF-β engage Naïve T cells to differentiate into the Th17 cells phenotype [36]. Then, IL-23 stabilizes the engaged Th17 cells into a pathological phenotype [36]. Here, we showed that AChR$^+$ MG TECs displayed an increased expression of cytokines involved in the activation of the IL-23/Th17 pathway. These results are in line with various studies that have shown implication of pathogenic Th17 cells into inflammatory processes that occurred in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and Psoriasis [30,37]. Therefore, autoimmune MG shares with others autoimmune diseases the sameIL-23/Th17 activation pathway, in its disease effector tissue, the thymus.

Of note, IL-23 is constituted of two subunits IL-23p19 and IL-12p40 [38]. IL-12p40 favors the differentiation of Th1 cells that produce IFN-γ [39] while IL-23p19 subunit is responsible for the Th17 cell pathogenicity through the stabilization of the phenotype and consequent IL-17 secretion [40]. Depletion of IL-12p40 and IFN-γ does not protect mice from the development of experimental autoimmune myasthenia gravis mouse model (EAMG) based on active immunization, a mouse model that does not involve thymic inflammation [41]. However, our data demonstrate that AChR$^+$ hyperplastic MG thymuses harbor an IL-23p19 over-expression. Interestingly, in experimental autoimmune encephalomyelitis, multiple sclerosis mouse model, stronger disease symptoms occur when Th17 cells are developed in presence of IL-23 [35]. An effect corroborated by the resistance to the disease induction in IL-23p19$^{-/-}$ mice [42].

Numerous studies have highlighted the heterogeneity of Th17 cells [43,44]. Th17 differentiation into pathogenic and non-pathogenic cell subgroups have been shown to rely on the cytokine combination or the pathogens used for the differentiation [13]. More, pathogenic and non-pathogenic Th17 cells have been identified with specific gene signature (IL-10, IL-9, Aryl hydrocarbon receptor, Maf for non-pathogenic cells; IL17R, GMZB, IL-22, Stat4, IL-23R for pathogenic subsets [23]). Given the phenotypical gene signatures and component milieu identified to describe the complex Th17 cells, our study shows that in the AChR$^+$ MG thymuses, TECs over-produce IL-23 and TGF-β3 in addition to IL-6, and IL-1β [30,37], a pro-inflammatory context that contributes to the differentiation and activation of pathogenic Th17 cells, emphasized by the IL-23R increased expression found in MG thymocytes.

4.2. Thymic extrinsic factors responsible for mTECs to sustain Th17 prone milieu

IL-23 expression is induced by various factors such as TNF-α, IL-1β and IFN-γ through the NF-κB pathway in dendritic cells, macrophages and keratinocytes [21,45]. In MG thymus, inflammatory factors such as IFN-γ and TNF-α are known to be overexpressed [46,47]. Poly (I:C) activated IFN-I pathway in TECs induced an overexpression of the α-AChR subunit (normally expressed in the thymus as a tissue specific antigen), the IFN-I production [25] and stimulates respectively in TECs and lymphatic endothelial cells, CXCL13 and CCL21 expression, two B-cell chemoattractant proteins involved in eGC development in MG thymuses [48]. Here, we have added IL-23, as another target of IFN-I transduction pathway in MG TECs. Deregulation in the IFN-I pathway is possibly a critical factor that activates inflammatory signaling in MG TECs and induces the expression of IL-23 among other cytokines.

AChR$^+$ MG with hyperplastic thymus is a "female" pathology [1]. Estrogens may then play different roles in this disease, by facilitating the autoimmune reaction though a defective tolerance process [19] and by modulating the basal level of IFN-I [48]. In AChR$^+$ MG thymus, an activation of IFN-I may be potentialized by estrogen receptor-α signaling, contributing then to induce the production of IL-23 in TECs. Estrogens can also affect IL23R expression, the production of IL-17 and the percentage of Th17 cells [49]. Therefore, AChR$^+$ MG female may challenge a synergy of an activated pathway that shifts towards a Th17 inflammatory status in the thymus.

4.3. MG TECs contribution in eGC formation

A common characteristic of autoimmune MG with other organ-specific autoimmune diseases is immune cells infiltrations (i.e. B cells)

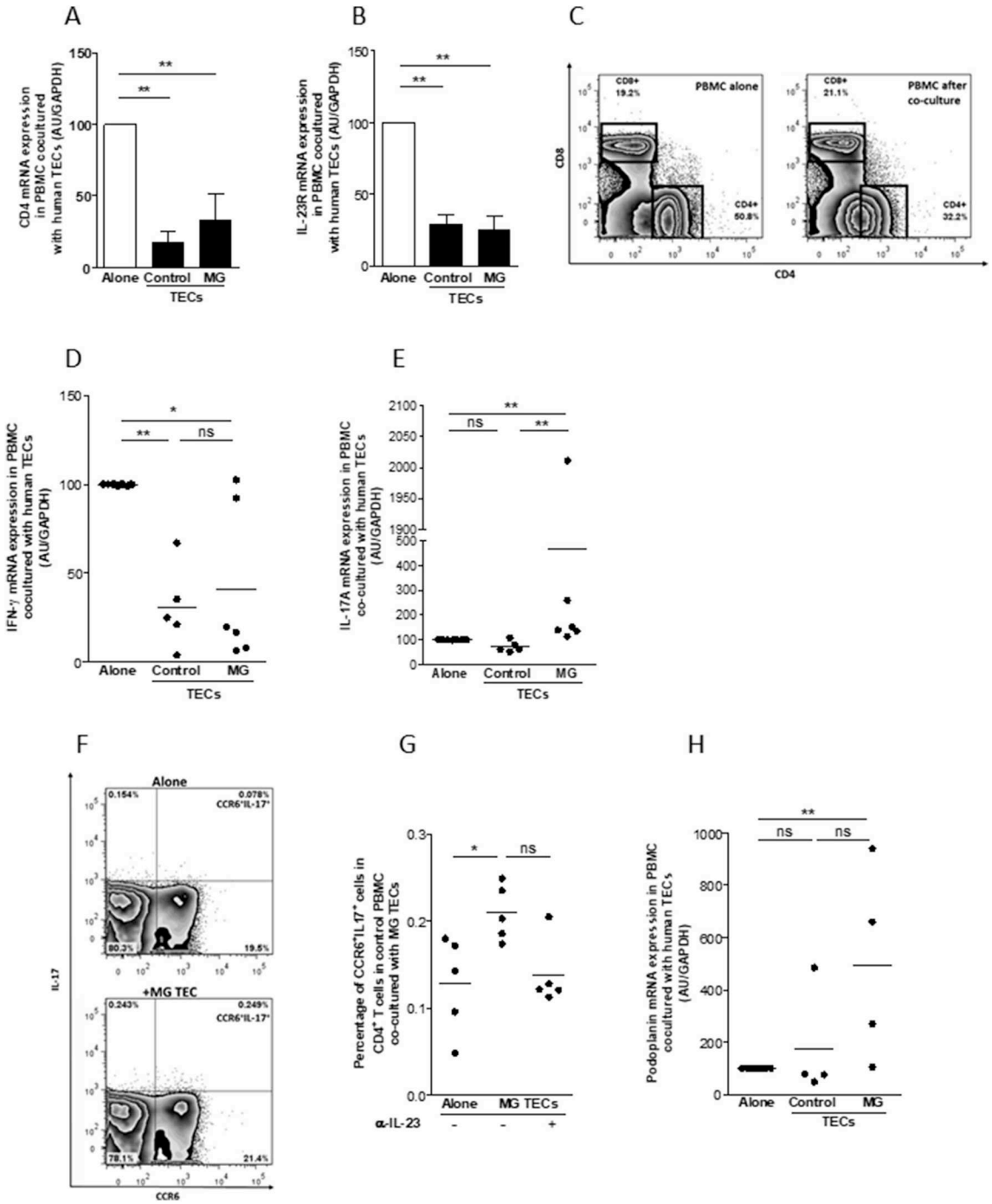

Fig. 5. MG TEC secretion of IL-23 stimulates IL-17 production by PBMCS. Analysis of the mRNA expression of **A)** CD4 and **B)** IL-23 receptor in PBMCs co-cultured for 24 h with controls or AChR$^+$ MG primary TECs. **C)** Representative images of flow cytometry analysis of CD4 and CD8 labelling of PBMCs cultured with or without control TECs. mRNA expression levels of **D)** IFN-γ and-**E)** IL-17A in PBMCs co-cultured for 24 h with control and AChR$^+$ MG TECs. **F)** Representative image of flow cytometry analysis of CCR6$^+$ IL17$^+$ cells in control PBMCs cultured with or without AChR$^+$ MG TECs. **G)** Flow cytometry analysis of the percentage of CCR6$^+$IL-17$^+$ cells among the CD4$^+$ cells in PBMCs co-cultured with AChR$^+$ MG TECs and an antibody anti-IL-23. **H)** mRNA expression level of Podoplanin in PBMCs co-cultured with control or AChR$^+$ MG TECs. mRNA expression was analyzed by real-time PCR and normalized to GAPDH. mRNA expression is expressed as arbitrary unit (AU). Each point represents the mean value of duplicate analysis of different PBMC donor. n > 4 PBMCs from different controls. Primary cultured human TECs were obtained from N > 3 different individuals for controls and AChR$^+$ MG patients. P values were obtained using ANOVA analysis or non-parametric *t*-test (*p < 0.05; **p < 0.01; ***p < 0.0005).

in the inflamed tissues [50]. In MG thymuses, TECs attract B cells via CXCL13 expression [10] that could contribute to eGC development. In addition here, we showed that in thymic eGCs, CD4$^+$ cells expressed IL-17 and PDPN. Then our observations may suggest that Th17 cells that develop in the presence of IL-23 participate in the maintenance of eGCs in MG thymuses through PDPN as shown in other pathologies [14,51]. More, the overexpression of IL-21 in MG thymus, a cytokine expressed by Th17 cells (among other cells) and involved in the differentiation of B cells into plasma cells reinforces the potential role of the IL-23/Th17 pathway in MG thymic eGC homeostasis.

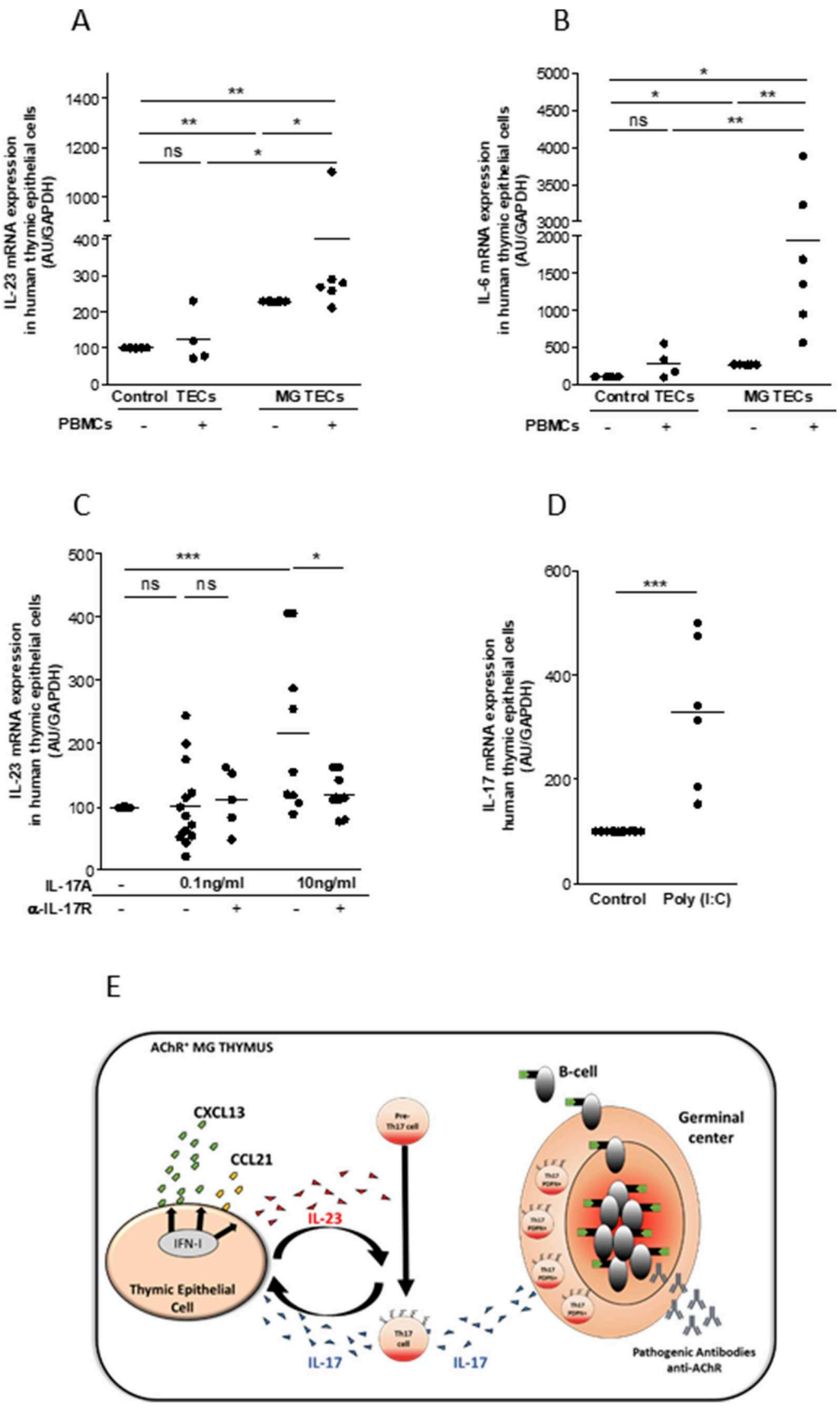

Fig. 6. PBMCs co-cultured with TECs influence the TEC expression of cytokines. mRNA expression level of **A)** IL-23 and **B)** IL-6 in control and MG AChR$^+$ TECs co-cultured with control PBMCs. **C)** Effect of IL-17 (0.1 ng/ml and 10 ng/ml) and anti-IL-17 receptor (α-IL-17R) (1 μg/ml) on the mRNA expression of IL-23 by control TECs. **D)** Effect of Poly (I:C) on the mRNA expression level of IL-17 by control TECs. **E)** Schematic representation of IL-23/IL-17 loop of regulation in AChR$^+$ MG thymuses. mRNA expression was analyzed by real-time PCR and normalized to GAPDH. mRNA expression is expressed as arbitrary unit (AU). Each point represents a different donor. N > 4. Primary cultured human TECs were obtained from n > 5 different individual controls. P values were obtained using ANOVA analysis or non-parametric *t*-test (*p < 0.05; **p < 0.01; ***p < to 0.0005).

4.4. Why does the inflammatory process do not stop?

The immune system relies on the capacity of cells to induce, when needed, inflammation and to resolve it. Similarly, to other autoimmune diseases, AChR$^+$ MG patients face a chronic inflammation. One can wonder why the immune system is powerless to resolve the intra thymic inflammation. Some clues have emerged from our data and previous studies.

First of all, AChR$^+$ MG thymic Treg cells display a defective capacity to suppress effector T cell proliferation [6]. In parallel, thymic effector CD4$^+$ T cells are not responsive to suppression. The increased percentage of activated IL-17 expressing cells in MG thymus may be a consequence of the impaired ability of Treg cells to suppress effector T cells and to resolve inflammation. Moreover, Treg cells can mimic Th17 cells [52] in the presence of increased IL-23 medium level. Therefore, plastic Treg cells in AChR$^+$ MG tend to become Th17-like ex-Treg cells [8] and contribute to rise IL-17 concentration in the thymus.

IL-17 activates in stromal cells the NF-κB pathway and induces the expression of pro-inflammatory molecules like IL-6, IL-8, GM-CSF and CCL20 [53,54]. As our results show, MG TECs are also responsive to IL-17. Therefore, we can envisage that MG TECs have a double side participation in the inflammatory process. One side by inducing the production of IL-17 by effector and regulatory T cells while on another side by sensing IL-17, amplifying the inflammatory signal and starting an unstoppable inflammatory process.

5. Conclusions

Within these settings, we propose that activation of the IL-23/Th17 pathway engenders an endless cascade of signals that might be triggered by a deregulation of the IFN-I pathway (Fig. 6E). In addition, the IFN-I pathway induces expression of chemokines promoting B-cell infiltration as well as of pro-inflammatory cytokines involved in the differentiation of pathogenic Th17 cells expressing PDPN. These pathogenic cells might collaborate in the inflammatory process by sustaining the formation of eGCs through IL-21 signaling, and also by stimulating and amplifying IL-23 expression by TECs through IL-17 signaling retro positive control.

Altogether, our study reveals a new promising therapeutic target for AChR$^+$ MG patients, the IL-23/Th17pathway. To date, monoclonal antibodies anti-IL-23 (such as Ustekinumab) are already approved by FDA and are used to treat patients affected with Crohn's disease and psoriatic arthritis [55]. Therefore, we consider that targeting IL-23 in AChR$^+$ MG could decrease concomitantly thymic and peripheral inflammation and antibody production. Therefore, IL-23 should be proposed as a new promising therapeutic target in MG.

Authors contributions

J.V., J.B. and N.D. performed the experiments, analyzed the data and interpreted the results. F.T. provided help to obtain samples. I.K. and AC.B. performed the podoplanin experiments. R.R. and N.S provided human thymic tissues. R.L.P. provided samples and helpful suggestions to design experiments. S.B-A. initiated the study. N.D. and S.B-A were involved in all aspects of the study including: design, data analysis and interpretation of the results. N.D. and J.V. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Declaration of interest

The authors declare that they have no relevant conflicts of interest.

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Article #3

IL-23 targeted treatment prevents and ameliorates symptoms in humanized and classical mouse models of myasthenia gravis.

Article in preparation

Objective

The main objective was to find a targeted therapy that may cure the inflammation and the muscle disturbances observed in AChR$^+$ MG patients. To do so, we have used two efficient mouse models specifically to investigate the therapeutic impact on the thymus (NSG-MG mouse model) and the muscle (EAMG mouse model).

The preclinical humanized mouse MG model has been developed in the lab to test novel therapies. AChR$^+$ MG thymic biopsies are engrafted subcutaneously to immunodeficient NOD SCID gamma (NSG) mice (NSG-MG). Twenty to 30 days after engraftment, the humanized NSG-MG model recapitulates thymic inflammation, antibody production, Th17 cell activation and muscle symptoms characteristics of MG. This is a good preclinical model for analyzing whether a thymic cure can alleviate clinical MG symptoms.

The EAMG mouse model is characterized by a high concentration of anti-AChR autoantibodies and provides an excellent model to analyze the effects of the autoantibodies in muscle physiology. It is also an efficient model to analyze the impact of inflammation and antibodies on muscle physiology.

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IL-23 Targeted Treatment Prevents and Ameliorates Symptoms in Humanized and Classical Mouse Models of Myasthenia Gravis

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Abstract

AChR$^+$ Myasthenia gravis (MG) is a chronic autoimmune disease characterized by thymic hyperplasia. MG thymus is known to present an overexpression of cytokines like IL-6, IL-1β, TGF-β1/3, and IL-23 that favors the Th17 cells development. To date, the therapeutic approaches of MG patients do not provide a cure and present many secondary effects. Thymectomy has been shown to be effective to reduce clinical symptoms, however most patients remain under corticosteroid treatment.

Here, we aimed to study a potential novel therapeutic approach that targets IL-23p19 (IL-23) in the humanized MG mouse model based on engrafted human MG thymuses on NSG mouse (NSG-MG) and in the classical experimental MG mouse model based on immunization of C57BL6 mouse with the acetyl choline receptor obtained from torpedo fish (EAMG), the two main pre-clinical MG models. We determine in NSG-MG mouse model the impact of anti-IL-23p19 on the inflammation observed in human MG thymus. We analyzed the impact of anti-IL-23 on the production of anti-TAChR antibodies in the EAMG model and evaluated the repercussion in muscle physiology.

In both preclinical models, anti-IL-23p19 treatment induced an amelioration in the clinical symptoms. In the NSG-MG model, anti-IL-23 reduced the levels of IL-17 and TGF-β3, as well as the percentage of pathogenic Th17 cells in the human grafted MG thymus. Anti-IL-23 did not alter Treg cells compartment but reduced the expression of Activation-induced cytidine deaminase (AID) and podoplanin, two critical factors involved in B cell development and eGC formation, respectively. In the EAMG mouse model anti-IL-23p19 reduced the production of IL-17 and improved the Th17/Treg cell balance. Moreover, we observed a reduction in anti-TAChR antibody production and consequently a decreased in the activation of muscle satellite cells (SC) in mice receiving anti-IL-23 treatment.

These data indicate that targeting IL-23 with a monoclonal antibody can modulate the inflammatory state in MG thymus while reducing the production of antibodies and consequently ameliorate clinical symptoms of MG.

INTRODUCTION

Myasthenia gravis (MG) is a rare autoimmune pathology characterized by an alteration of thymus physiology [1]. In the most common form of autoimmune MG, patients have antibodies directed against acetylcholine receptors (AChR$^+$ MG) and thymic alterations such as hyperplasia or thymoma. Hyperplastic AChR$^+$ MG thymuses display ectopic germinal centers (eGC) [1] where B-cells produce AChR antibodies. MG hyperplastic thymuses also harbor an upregulated expression of B cell chemo-attractants, chemokines CXCL13 and CCL21 [2, 3] and cytokines such as IFN-I, IL-1β, IL-6, TGF-β1 [4, 5] [6, 7]. We have previously demonstrated that AChR$^+$ MG thymuses challenge a loop of inflammation, a result of elevated IFN-I pathway activation [4], and a T-cell imbalance [8, 9]. In addition, we have recently shown that the IL-23/Th17 pathway is active and participates in the perpetuation of the thymic inflammatory status [10]. Indeed, medullary thymic epithelial cells (mTEC) in AChR$^+$ MG thymus overexpress IL-23, that in addition to IL-1β, IL-6 and TGF-β1 promotes the differentiation of pathogenic Th17 cells. As a loop, over-activated Th17 cells sustain the MG TEC IL-23 overexpression [10].

IL-23/Th17 cell pathway is critical in and for the development of autoimmune diseases like multiple sclerosis, psoriasis and rheumatoid arthritis [11, 12]. Th17 cell differentiation and maturation are two steps carried out by the cytokine environmental content [13]. CD4$^+$ T-cells undergo a differentiation first step process sustained by IL-6 and TGF- β1, followed by a Th17 cell activation induced by IL-23 and TGF-β3 [14].

IL-23 is a dimeric cytokine composed of two subunits, IL-23p19 and IL-12p40, a subunit shared with IL-12 [15]. In experimental autoimmune encephalomyelitis (EAE), the mouse model of MS, IL-23-differentiated Th17 cells overexpressed IL-17 and podoplanin and are capable to promote the formation of eGCs [16]. More, IL-23-differentiated Th17 cells may overexpress IL-21 [17], an inflammatory cytokine that regulates expression of β-galactoside α2,6-sialytranferase1 (ST6gal1) [18], that is an enzyme that controls antibody pathogenicity by transferring sialic acid to immunoglobulins (IgG) in antibody producing cells [19]. In addition to their roles in IgG production components or systems, Th17 cells sustain the activation of inflamed stromal cells in lymph nodes [20]. Hence, pathogenic Th17 cells and

their related cytokines have been considered as new potential therapeutic target to alleviate and treat various autoimmune diseases [21]. To date, clinical trials have shown that blocking different actors of the IL-23/Th17 pathway especially IL-23 can ameliorate some psoriasis patients [22].

To date, there is no cure for AChR^{+} autoimmune MG. Treatment of myasthenia gravis relies in three types of non-exclusive therapies. The first includes symptomatic therapies with anticholinesterase, plasma exchange and intravenous immunoglobulins that permit a rapid relief of clinical symptoms of patients but without an effect in the immunopathology [23]. The second type of therapy is long-term treatment with corticosteroids and immunosuppressors [24]. Although these long-term therapies can be efficient in a considerable number of patients they present challenges due to the many side effects and comorbidities [24, 25]. Another therapeutic option is thymectomy that is an effective therapy to ameliorate MG symptoms. However not all patients are eligible, and most patients still required long-term glucocorticoid therapy [26]. Hence, the need of novel therapies that could alleviate and stop the chronic inflammation and their consequences remains.

With the aim to ameliorate the disease outcome, we wondered whether stopping (or reducing) the thymic inflammation and the associated disturbance could be a new therapeutic option. To this end, we targeted IL-23p19 in the two relevant experimental mouse models of MG, the humanized MG model based in the subcutaneous engraftment of MG thymus in immunodeficient mouse (NSG-MG)[27], and the classical experimental autoimmune MG (EAMG)[28] focusing on the impact of autoantibodies on the muscle.

MATERIALS & METHODS

Human samples

Control human thymuses were obtained from patients undergoing cardiac surgery at the hospital Marie Lannelongue Chirurgical Center (Le Plessis-Robinson, France). Myasthenia gravis thymic biopsies were obtained from patients undergoing thymectomy at the Marie Lannelongue Chirurgical Center (Le Plessis-Robinson, France) and at the Strasbourg civil hospital (Strasbourg, France). 4 MG thymuses aged of 22 to 26 years old were included. Written informed consent was obtained from all donors or the legal representant. This study was approved by the local ethics committee (CPP, Kremlin-Bicêtre, France: agreement N°06-018; CCP Ile de France Paris 7, France agreement N°C09-36). Clinical characteristics of MG patients are presented in **table 1**.

NSG-MG mouse model

NOD-SCID IL-2Rγ null (NSG) mice were obtained from Charles Rivers laboratories and kept in our facilities under specific pathogen-free conditions. Mice aged of 8-12 weeks old were engrafted with freshly recovered human thymic biopsies subcutaneously through a minimal incision. Four to six pieces of thymic biopsies were engrafted in the mouse back. We followed the protocol described and established by Sudres et al [27]. Since no difference in disease development, in NSG MG model, was observed by using prednisone treated MG patients, we engrafted thymic biopsies regardless the prednisone patient status [27]. NSG-MG mice were treated by intraperitoneal injection with 100µg/mice/week of monoclonal anti-human IL-23p19 antibody (Clone HNU2319, Thermo-fisher). Treatment was given for 4 weeks starting at day 15 after engraftment (period of clinical MG symptoms arise [27]). Control mice were treated with an equivalent volume of physiological serum (Ab diluent). At day 42, mice were euthanized and xenogeneic human thymi, spleen, blood and muscle (Tibialis anterior) were recovered and used freshly or stored at -80^{o}C for later analysis.

This study was approved by the French ministry of agriculture committee for the animal used (Authorization number # 02622-22). All animals were handled according to the Animal

Care and Use of Laboratory Animal guidelines and in a facility approved of the French Ministry of Research (Authorization number B-75-13-20).

Experimental autoimmune myasthenia gravis (EAMG)

Five weeks old female C57Bl/6 mice were obtained from Janvier laboratories (Le Genest Saint-Isle, France) and placed in our animal facilities for 1 week. Then mice were immunized as previously described by Tuzun et al [28]. Briefly, mice were injected subcutaneously in hind footpads and in the back with an emulsion containing purified torpedo acetylcholine receptor (TAChR) (30µg), Complete Freund Adjuvant (CFA) (Sigma, Saint Quentin Fallavier, France) and non-viable Mycobacterium tuberculosis (1mg/mouse) (BD Difco, Villepinte, France). Control mice were injected with an emulsion containing only CFA and non-viable mycobacterium tuberculosis. Three weeks after the first immunization, mice received a second immunization with a solution containing CFA and TAChR or CFA only. EAMG mice were treated by intraperitoneal injection with 100µg of monoclonal anti-mouse IL-23p19 antibody (Clone G23-8, Thermo-fisher). Treatment was started 2 weeks after the second immunization with a weekly dose for either 2 or 4 weeks. One week after the last treatment mice were euthanized. Blood, spleen and muscle (Tibialis anterior) were recovered and used freshly or stored at -80^{o}C for later analysis.

This study was approved by the French ministry of agriculture committee for the animal used (authorization number #3692-2016012111336184). All animals were handled according to the Animal Care and Use of Laboratory Animal guidelines and in a facility approved of the French Ministry of Research (authorization number B-75-13-20).

Clinical test

A complete clinical analysis of each mouse was done weekly as previously described [29] and following the protocol and recommendations established by Losen et al [30]. Animals were weighted and checked for signs of fatigue or unusual behavior (Ears and/or tail down, abnormal movements or reduced mobility). To determine the clinical disease score, we performed the grip test (after treadmill exercise) and hire test. The different tests combined

together provided a disease score between 0 (not sick) to 9 (dead or euthanized before end of the experiment) [31].

RNA extraction and reverse transcription

Frozen human thymus and mouse spleen and tibialis anterior (TA) were homogenized with the FastPrep FP120 instrument (Qbiogen, Illkirch, France). Total RNA was extracted from the thymus, spleen or muscle using the Trizol RNA Isolation kit (Invitrogen, Cergy-Pontoise, France). Total mRNA (1µg) was reverse transcribed using the AMV first strand cDNA synthesis kit (Roche diagnostics, Meylan, France) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Gene expression was evaluated by quantitative real-time PCR performed using the Light-Cycler apparatus (Roche Diagnostics; Meylan, France) as previously described by Dragin et al (Dragin et al. 2016). Each PCR was performed, in duplicate, using the Fast-start DNA Master SYBR Green I kit (Roche Diagnostics; Meylan, France) according to the manufacturer's instruction. Each cDNA sample was run in duplicate. Samples were normalized as specified in the figure legends. The list of primers is summarized in **table 2**.

Human ELISA

The levels of human cytokines IL-2, IL-6, IL-17, IL-21, TGF-β1 and TGF-β3 were evaluated on extracted proteins. Total tissular proteins were extracted with a solution containing 5% Tris HCl 20mM, 0.1% Triton X100, and one tablet of protease inhibitor cocktail (Roche-Diagnostics, Meylan, France) using the fast prep apparatus. All ELISA kits were obtained from R&D systems (Lille, France). Each ELISA was performed in duplicate and according to the manufacturer's instructions. ELISA reactions were read with a SPARK ELISA microplate reader (Tecan, Männedorf, Switzerland).

Detection of human IgG and anti-AChR antibodies

Quantification of circulating total human IgG in NSG mice was done by ELISA. Polyclonal rabbit anti-human IgG was incubated overnight in 96 wells plate at 4°C. Next, for 1 hour at

room temperature, a blocking buffer is added (1% PBS-BSA) following two PBS wash steps. Then 100µl of serum and standards were added and incubated for 1 hour. Next, a HRP-coupled polyclonal rabbit anti human IgG was added for 2h at 37°C. The revelation was done with 3, 3', 5, 5' tetramethyl benzidine (Sigma-Aldrich, Lyon France). Reaction was stopped with H_3PO_4. Absorbance was measured at 450nm. The antibodies used for human total IgG were purchased from DAKO (Courtaboeuf, France).

Detection of human anti-AChR antibodies in engrafted NSG mouse serum was done using the AChR autoantibody ELISA kit from RSR (RSR limited, United Kingdom) accordingly to manufacturer's instructions. Briefly, 100µl of serum was incubated with a mixture of fetal and adult AChR overnight at 4°C and coated the day after in ELISA plate for 1 hour. Then a biotinylated anti-AChR Ab was added and revealed with streptavidin-HRP and TMB (Sigma-Aldrich, Lyon France). Optical density (OD) was determined at 450 nm in SPARK ELISA microplate reader (Tecan, Männedorf, Switzerland). Concentration of human anti-AChR was inversely correlated to the absorbance obtained at 450 nm.

Detection of anti-TAChR antibodies

96-well ELISA plates were coated overnight at 4°C with 1µg/ml of T-AChR diluted in 10 mM NaHCO3 buffer, pH 9.6. The day after, the ELISA plate with coated TAChR was blocked with 10% SVF in PBS at 37°C for 2 hours. Then, 100 µl of mouse serum (dilution 1/100,000) were added and incubated for 2h at 37°C. After washing with PBS-Tween buffer, we completed the ELISA sandwich by adding 100 µl of biotinylated anti-mouse IgGs (dilution 1/10,000) (Dako, Courtaboeuf, France). The plate was incubated 2h at 37°C. The plate was then washed and incubated with 100 µl of streptavidin-horseradish peroxidase (Dilution 1/20,000) (Life Technologies, Courtaboeuf, France) for 30 minutes and revealed with tetramethylbenzedine. Optical density (OD) was determined at 450 nm in SPARK ELISA microplate reader (Tecan, Männedorf, Switzerland).

Flow cytometry analysis

To analyze the circulating human cells content in NSG-mice, fresh blood was taken once a week. After centrifugation, serum was recovered and frozen at -80°C. Lymphocytes were

obtained after lysis of red blood cells with BD lysing buffer (BD biosciences) for 10 min and stained with anti-human antibodies against CD45, CD4, CD8, CD19, CCR4, CCR6, IL-23R, CD25 and CD127 (BD bioscience, Le Pont de Claix, France). The antibodies used for this analysis are listed in **table 3.** Cells were acquired in FACS Canto II Analyzer (BD) and analyzed using FlowJo software (Tree star, Olten, Switzerland).

At the sacrifice, pieces of xenogeneic human thymi and mouse spleens were mechanically dissociated in PBS to obtain a cell suspension. The cell suspensions were labelled as previously described for the blood cells

Immunohistochemistry analysis

Cryostat sections (7µm thick) of xenogeneic human thymi or mouse spleens were fixed with acetone to glass superfrost slides and dried for 1h. Slides with tissue sections were pre-incubated with a blocking buffer (PBS, 0.1% BSA, 10%FBS, 0.3M glycine, 1% Tween) for 1h at room temperature. Then, they were incubated overnight at 4°C with antibodies raised against human or mouse antigens. Revelation was done with Alexa 488, Alexa 594 or Alexa 350 couples secondary IgG raised in Rabbit, chicken or donkey. Images were acquired with a Zeiss Axio observer Z1 inverted microscope using 20x magnification (Carl Zeiss, Le Pecq, France). The antibodies used for this analysis are listed in **table 3.**

Statistical analysis

Non-parametric tests (Wilcoxon test for paired data or Mann-Whitney test for unpaired values) were used to compare groups as specified in each figure legend. Values were reported as Mean ± Sem. GraphPad Prism 5 software was used to generate the graphs and to perform the statistical analysis. Statistical significance was recognized at $p < 0.05$.

RESULTS

Sudres et. al. have previously shown that engrafted NSG mice with MG thymuses (NSG-MG) mimic different MG features observed in human including increased inflammatory markers (T-cells, cytokines) in the circulation and the spleen as well as the presence of circulating autoantibodies anti-AChR and myasthenic symptoms [27]. One of the key points for the success of the humanized MG mouse model is the vascularization of the human thymic biopsies and the subsequent release from the thymus of human lymphocytes and immunoglobulins.

We first determined whether our treatment with monoclonal antibody anti-IL-23p19 alters the general physiology of the engrafted human thymic biopsy. Immunohistochemical analyses revealed that murine vascularization observed by the presence of murine endothelial cells (mCD31-green) was not affected in mice receiving the therapy anti-IL-23 in recovered MG thymic biopsies in compare to controls **(Figures 1A and 1B).** As well, we analyzed the content of the main thymic cell populations by flow cytometry. As shown in **Figures 1C, 1D and 1E,** the treatment with anti-IL-23p19 did not alter the thymic percentage of CD4sp, CD8sp or $CD19^+$ cells suggesting that no change in the global T and B cell population repartition is induced. Furthermore, engrafted human thymuses should release immunoglobulins and cells into the mouse blood. Thus, we determined the impact of the treatment on the release of human immunoglobulins in mouse blood weekly from day 7 up to the sacrifice time (42 days after the engraftment). Our result showed no alteration or modifications in the total IgG release **(Figure 1F),** neither in the T cells and B cells export to the periphery **(Figures 1G and 1H)**. More, Sudres et. al. have previously shown that human lymphocytes released by MG thymuses are able to home into the mouse spleen [27]. We investigated whether the treatment may alter this process. At day 42 after engraftment, the spleen was analyzed. The treatment induced no significant difference in their weight **(Figure 1I)**, in the percentage of immigrant human $CD4^+$ or $CD19^+$ cells **(Figures 1J and 1K)** nor in the mRNA and protein expressions of human KI67 (marker of global cellular proliferation of human cells) **(Figures 1L, 1M and 1N)**. Altogether these data demonstrate

that treatment did not induce thymic physiological changes neither cell export dysfunctions nor deregulations.

We have previously showed that AChR$^+$ MG thymuses present an increased expression of IL-17, as well as the cytokines required for the differentiation of Th17 cells (i.e., IL-6, TGF-β1/3, IL-21 and IL-23) [10]. The treatment of NSG-MG mice with a monoclonal antibody anti-human IL-23p19 did not modify the engrafted thymic level of IL-6 and TGF-β1, cytokines involved in the initial development of Th17 cells **(Figures 2A and 2B)**, neither IL-21 expression, a cytokine required for the differentiation and the regenerative feedback mechanism for Th17 cells **(Figure 2C**). However, we observed changes in the Th17 cell induction compartment [32]. TGF-β3, a cytokine induced by IL-23 and involved in the development of pathogenic Th17 cells [14] showed a consistent decreased thymic expression in mice receiving the treatment with anti-IL-23p19 **(Figure 2D)**. Similar result was obtained for the thymic level of IL-17A, the classical cytokine produced by Th17 cells **(Figure 2E)**. These observations were corroborated by a significant reduction in the thymus of the percentage of two subpopulations of human Th17 cells characterized as CD4$^+$CCR6$^+$CCR4$^+$ cells and CD4$^+$CCR6$^+$IL-23R$^+$ cells in NSG-MG treated mice **(Figures 2F and 2G)**.

We also checked the treatment impact on the Treg compartment. We observed no change on IL-2 thymic protein level **(Figure 2H)**, and on the thymic percentage of CD4$^+$CD25$^+$ CD127$^-$ cells **(Figure 2I)**. Finally, functional blockade of IL-23p19 did not affect the expression of IFN-γ **(Figure 2J)** suggesting that the effects of the anti-IL-23p19 treatment can specifically target the development of pathogenic Th17 cells and can restore an equilibrium between Th17 and Treg cells.

Altogether, these data show that the anti-IL-23 targeted treatment, may be active into the MG thymus by decreasing Th17 cell pathogenic and inflammatory markers without altering the Treg compartment.

AChR$^+$ MG thymus is characterized by an abnormal presence of B-cells organized in ectopic germinal centers. We have previously demonstrated that AChR$^+$ MG thymuses display an

increased expression of activation-induced cytidine deaminase (AID) (a protein involved in B-cell somatic hypermutation) and podoplanin (a protein stabilizing the ectopical germinal centers) [10]. We wondered whether the decrease in the Th17 cell compartment may also impact the eGCs and their related gene regulators. The analyses by RT-PCR of the expression of AID and podoplanin in MG engrafted thymuses showed a decrease in the treated group of NSG-MG mice **(Figures 3A and 3B)**. For the B-cell chemoattractant CXCL13, no significant change is observed **(Figure 3C)**. In addition, the treatment induced no change in B lymphocyte induced maturation protein-1 (Blimp1), an IL-21 target gene [33] and a protein involved in B cell maturation and activation [34] **(Figure 3D)**. More, we have analyzed the expression of ST6gal1, a type II membrane protein that catalyzes the transfer of sialic acid from CMP-sialic acid to galactose-containing substrates in IgG, decreasing then their pathogenicity and known to be regulated by the Th17/IL-23 pathway [18]. We observed a slight increase in the thymic expression of ST6gal1 although no significant **(Figure 3E)**. Finally, we corroborate that despite the subtle changes observed in B-cell compartment, the medullary zone of the thymus remained unchanged **(Figure 3F, 3G and 3H)**. Altogether these data suggest that anti-IL-23p19 does not reduce the chemoattraction of B-cells to the thymus but may reduce their activation and may prevent their organization in eGCs within the thymus.

We then wondered whether the reduction in the percentage of Th17 cells observed in the thymus could also be found in the circulation and the spleen. As shown in **figures 4A and 4B,** the treatment stimulates the decrease of circulating Th17 cells, even though no significant. More, as observed in the human thymic biopsies, treatment induces no modification in the percentage of circulating Treg cells **(Figure 4C)**. Moreover, in the spleen, we observed a significant decreased in $CD4^+CCR6^+CCR4^+$ T-cells **(Figure 4D)** but only a slight decrease in those identified as $CD4^+CCR6^+IL\text{-}23R^+$ **(Figure 4E)** or Treg cells **(Figure 4F)**.

Furthermore, we quantified in serum of NSG-MG mice the concentration of human anti-AChR antibodies and we did not find a significant decrease **(Figure 4G)**. Nevertheless, it is

known that the concentration of autoantibodies is not correlated with the disease outcome [35].

In order to corroborate the effect induced by the anti-IL-23p19 treatment on the NSG-MG humanized model, we tested an antibody homologue anti-mouse anti-IL-23p19 on the classical experimental autoimmune myasthenia gravis model (EAMG). We first observed a similar and significant decrease in the circulating IL-17 level **(Figure 5A).** Since inflammation is associated with the disequilibrium between Th17 and Treg cells, we also checked in the EAMG model the circulating T-cell population composition. We observed only a slight decrease in the percentage of Th17 cells ($CD4^+CD62L^-CCR6^+$) of mice treated with anti-IL-23p19in the treated mice **(Figure 5B)**. Interestingly, the Treg cells population ($CD4^+CD25^+CD127^-$) in EAMG mice showed a significant decrease compared with controls as previously reported [36]. However, mice treated with anti-IL-23p19 displayed an increase although not significant in this population **(Figure 5C).** Therefore, in EAMG mice, anti-IL-23 treatment modulates the circulating inflammatory status, which might be controlled by Th17/Treg cells equilibrium.

To determine whether the reduction of the inflammation had an impact on the antibody (Ab) production in EAMG, we analyzed their Ab content and subtype by ELISA. We observed that even though the total IgG content was not modified by the treatment **(Figure 6A),** the IgG1 and IgG2b isotypes, the pathogenic subtypes were significantly reduced in the anti-IL-23 treated group **(Figures 6B and 6C)**. Of note, corroborating what has been observed in the NSG-MG model, spleens of EAMG mouse model treated with anti-IL-23 decreased their expression of podoplanin and AID, factors involved in the formation/stabilization of the GCs **(Supplementary figure 1).**

Myasthenia gravis main clinical symptom is muscle fatigue. EAMG, is a mouse model that mimics the muscle symptoms of patients resulting from the attack of the anti-AChR

antibodies at the neuromuscular junction. In humans, the pathogen effects of the antibodies induce a reduction of AChR at the neuromuscular junction as well as an activation of satellite cells [37, 38]. More, we have previously reported that anti-AChR antibodies are capable to activate muscle satellite cells in EAMG mice [38].

Since our treatment with anti-IL-23p19 reduced the concentration of anti-TAChR antibodies, we wondered whether the reduction of pathogenic antibodies led to less activation of muscle satellite cells. As shown in **Figure 7A** the expression of Pax7 in non-treated EAMG mice is significantly higher compared to controls. Interestingly, EAMG mice treated with anti-IL-23p19 showed a significant reduction of muscular Pax7 expression **(Figure 7A)**. Moreover, after activation, satellite cells differentiate into myoblasts expressing MyoD. Therefore, we analyzed the expression of MyoD in our mouse model. Similar to Pax7, **Figure 7B** shows that indeed, non-treated EAMG mice presented a significant increased muscle expression of MyoD while the treated mice showed a reduced MyoD expression to the level of the control group **(Figure 7B)**. We then analyzed the expression of MyoG, a transcription factor marker of myoblasts engagement into a myogenic lineage [39]. **Figure 7C** shows that MyoG is overexpressed in muscle of treated EAMG mice **(Figure 7C)**.

We then investigated the possible mechanisms involved in skeletal muscle regeneration after attack by antibodies anti-AChR. Since TGF-β1 has been shown to promote myotube formation [40], we analyzed the muscle expression of TGF-β1 in EAMG mice. As observed in **figure 7D,** we found that muscle from non-treated EAMG mice had an important reduction in the expression of TGF-β1 compared to controls. This reduction was reversed by the anti-IL-23 treatment **(Figure 7D)**. More, IL-6 signaling has been involved in the activation of satellite cells [41]. Therefore, we analyzed the expression of IL-6 and IL-6R in skeletal muscle and found that, non-treated EAMG mice had a significant increased expression of IL-6 and IL-6R compared with controls **(Figures 7E and 7F)**. Interestingly, treatment with anti-IL-23 seems to modulate the expression of IL-6 but not IL-6R **(Figures 7E and 7F)**.

IL-17A has been shown to be overexpressed in inflammatory myopathies [42]. IL-17 in skeletal muscle has been shown to modulate myoblast differentiation [43]. Thus, we analyzed the expression of this cytokine in EAMG muscle. We observed a significant overexpression of IL-17A in non-treated EAMG mice compared with controls. Interestingly, mice receiving anti IL-23 showed a significant decreased IL-17 expression **(Figure 7G)** even though the cells producing IL-17 in the muscle remain to be identified.

These results suggest that treatment with anti-IL23p19 reduced markers of inflammation in the muscle which reduced the activation of satellite cells and allowed regeneration of skeletal muscle of EAMG mice.

In addition to modulate the expression of Th17 cells related pathogenic markers in thymus, blood and spleen, in NSG-MG mice model, the treatment affected the myasthenic muscle symptoms. As observed in **Figure 8A,** monoclonal antibody anti-IL-23p19 lowers significantly the disease clinical score. Interestingly, the decrease in clinical symptoms was observed one week after the initial dose and kept the same tendency up to day 42 (Area under curve (AUC) MG=25.69, MG treated=17.56) **(Figure 8A).**

As well, in the EAMG model, as shown in **Figure 8B,** treated mice required 2 weeks to reverse the slope of disease development (AUC MG=19.94; MG Treated =13.01) **(Figure 8B).** Of note**,** AUC analysis showed no significant change, although it decreased in 2 out of 3 independent experiments **(Figure 8B)**.

DISCUSSION

Th17 cells are known for their implication as drivers of autoimmunity. Development of Th17 cells is carried out by cytokines present in the microenvironment [14]. Among cytokines, IL-23 is a "game changer" when it comes to Th17 cell development. IL-23 is a cytokine that promote a pathogenic phenotype of Th17 cells that participate in development of pathologies like MS, RA and SLE [44-46].

We have previously shown that AChR^{+} MG thymus harbors an overexpression of IL-23 and an increased number of IL-17 producing cells [10]. Here, we took advantage of a recently established humanized mouse model of MG (NSG-MG) to show that specific targeting of human IL-23p19 with a monoclonal antibody reduced the inflammation related to pathogenic Th17 cells in human AChR^{+} MG thymus. In addition, we corroborate our results in the classical EAMG mouse model and evaluate muscle physiology in mice treated with anti-IL-23p19.

IL-17 related inflammation in EAMG mice

Analysis of IL-17 in MG patients have shown that autoreactive circulating T cells from MG patients produced high levels of IL-17, IFN-γ and GM-CSF [47]. More, levels of IL-17 in serum of MG patients has often been shown increased in compared to healthy controls [9, 48-50]. Even though, one study showed no difference in the production of IL-17 between PBMC of MG patients and healthy controls. This discrepancy may rely on ethnic, gender, age, treatment (effect of immunosuppressors) or methodology (analysis required activation of PBMC with anti-CD3) [51]. Moreover, we have provided evidence of a loop of thymic inflammation sustained by an increased thymic expression of IL-17 produced mainly by effector Th17 cells [10], and partially enhanced by inflammatory Treg cells [9].

The EAMG mouse model has been largely used to determine the mechanisms behind MG development through the use of mutant mice. For instance, Wei et. al. showed that IL-12/IL-23 p40 and IFN-γ double KO mouse was susceptible to develop EAMG [52]. In contrast, another report showed that IL-6$^{-/-}$ mouse was protected from development of EAMG [53].

More, mice that lack IL-17 were also protected from development of EAMG [54]. In both reports, mouse lacking IL-6 or IL-17 showed a reduced production of anti-TAChR antibodies. These reports sustain the hypothesis that Th17 cells contribute to the development of the disease. Our results with two different MG mouse models demonstrate and validate these hypotheses with a reduction of the circulating and tissue levels of IL-17 following a treatment that blocks IL-23p19.

Targeting pathogenic Th17 cells

IL-23 is a dimeric cytokine composed of IL-12p40 and IL-23p19. IL-12p40 is a shared subunit with IL-12p35 which is involved in Th1 development and production of IFN-γ [55]. IL-23p19 has been shown to specifically control the development of Th17 cells [56]. Therefore, we aimed to limit or to stop the thymic and peripheral inflammation by specifically blocking IL-23p19. We did not observe changes in the proportion of Treg cell population, or the cytokines required for their development (i.e. IL-2, TGF-β1), suggesting no alteration in the Treg cell compartment following the treatment. However, pathogenic Th17 cells ($CD4^+CCR6^+IL\text{-}23R^+$ cells) that required TGF-β3, in addition to IL-23 and expressed specific markers including IL-23R [14] are reduced. More, anti-IL23-p19 antibody altered IL-17 thymic, circulating and tissular content, without modification of the IFN-γ level. Therefore, it may be possible that treatment with anti-IL-23p19 may limit the Th17-related inflammatory status in $AChR^+$ MG thymuses by reducing pathogenic Th17 cells and improving the balance between inflammatory cells and Treg cells.

Th17 cells and eGCs

A hallmark of $AChR^+$ MG thymus is the presence of B-cell organized in eGCs. Pdpn is involved in the formation of GC in secondary lymphoid organs [57]. We have previously shown that $AChR^+$ MG thymus present T cells expressing Pdpn localized around thymic eGCs [10]. Peter et al. have demonstrated that T cells and mainly Th17 cells express Pdpn inside the eGCs in

the EAE mouse model. Moreover, they showed that blocking Pdpn in Th17 cells reduce the number of eGCs [16]. Th17 cells infiltrated in joints of an arthritic SGK mice model harbored also an overexpression of Pdpn [58]. Furthermore, in BXD2 strain mouse that spontaneous develops erosive arthritis and glomerulonephritis with presence of autoantibodies, IL-17 plays a critical role in the development of GC and the activation of B cells by inducing AID expression and the somatic hypermutation in B cells [59].

Here, in our two myasthenia gravis mouse models, we showed that blocking the production of IL-17 contributes to decrease Podoplanin and AID expression in the grafted thymus of NSG-MG mice and in the spleen of EAMG mice. Therefore, this suggests that as previously observed in other mouse models of autoimmune diseases, the decrease of the pathogenicity of Th17 cells might be translated into a reduction of eGC development and activation. More investigation should be done to validate this hypothesis. More, destabilizing the GC structure or components contribute to diminish the antibody production and consequently a decrease in the antibody attack.

Antibody production in EAMG and NSG-MG mice.

In MG the production of antibodies takes place mainly in the thymus. In EAMG, immunization with TAChR induces the production of antibodies within the secondary lymphoid organs. EAMG mice produce high levels of anti-TAChR antibodies. As previously mentioned, the production of antibodies requires the presence of the Th17-related cytokines [54, 60]. Our data showed that indeed, blocking IL-23p19 reduces the expression of IL-17 and leads to a reduced production of anti-TAChR antibodies. In contrast, in our NSG-MG mouse model, we did not observe a difference in the circulating antibodies in treated mice even if we did reduce the thymic expression of AID. A possible explanation is that the treatment effect on the thymic antibody producing cells and their secretion required a longer period of time compared to the effect on Th17 cells, and antibodies that are already produced and display a long lifespan in the periphery.

Muscle in EAMG

Myasthenia gravis main symptom is muscle fatigue due to the AChR degradation by autoantibodies. Many studies have shown the mechanisms by which anti-AChR antibodies change the physiology of the NMJ and the postsynaptic membrane [61, 62]. However, less is known about the consequences of anti-AChR antibodies within the skeletal muscle. Muscle is sensible to inflammation [63]. Indeed, IL-6, a proinflammatory cytokine can reduce AChR content and increase apoptosis of skeletal muscle cells [64]. Moreover, IL-6 can also be produced by skeletal muscle. For instance, anti-AChR antibodies promote the expression of IL-6 and IL-6 Receptor in skeletal muscle [65]. Chronic IL-6R activation in the muscle can lead to reduced Akt phosphorylation and promote muscular atrophy [66]. Interestingly, expression of IL-6 in skeletal muscle can promote activation of SC [41]. Our results in EAMG muscle demonstrate that by controlling the Th17 cells development, we may reduce the production of anti-AChR that targets the skeletal muscle and induce IL-6 expression.

Furthermore, targeting Th17 cells can regulate the activation of SC, critical actors of muscle regeneration. SC are stem cells that develop upon stimulation. SC present a differential expression of transcription factors throughout their differentiation into myotubes. For instance, after activation, SC express Pax7 and MyoD and start their proliferation. Then, to differentiate into myotubes, SC downregulate the expression of Pax7 and MyoD and up regulate MyoG [39]. Previously, Attia et. al. have shown that anti-AChR antibodies activate satellite cells in MG patients and EAMG mouse model. In this line, our results showed that by controlling inflammation in EAMG muscle, we decreased the activation of satellite cells and promote their myotube differentiation.

Targeting IL-23p19 reduced myasthenic clinical signs in NSG-MG and EAMG

In this study, we show for the first time that functional blockage of IL-23p19 with a monoclonal antibody ameliorates myasthenic symptoms in the classical and the humanized mouse models of Myasthenia gravis.

Monoclonal antibodies that target the IL-23/Th17 cells pathway are emerging as therapies to treat autoimmune diseases. For instance, ustekinumab, an anti-IL23p40 monoclonal antibody is now used for the treatment of psoriasis and Crohn's disease [67, 68]. Moreover, guselkumab, an anti-IL-23p19 monoclonal antibody is now approved to treat psoriasis [69].

Our results in NSG-MG pre-clinical model showed that mice receiving anti-IL-23p19 had a constant lower clinical score than mice that did not receive the antibody. Although the reduction of clinical symptoms was significant, the clinical scores of treated mice remained high compared with mice engrafted with non-MG thymus. This observation can be explained by the release of anti-AChR antibodies before the treatment. We treated NSG-MG mice at day 15 after the engraftment of the thymus when mice started to show myasthenic symptoms. Therefore, at this point mice already present circulating anti-AChR antibodies and our treatment did not aim to directly regulate antibody activity. However, it is possible that by increasing the dose we could still decrease the production of antibodies and to observe a substantial amelioration of clinical symptoms.

Moreover, the classical EAMG mouse model was also challenged with anti-IL23p19 and showed a reduction of clinical symptoms 2 weeks after the initial dose. At the end of the study and after 4 weeks of treatment, treated mice had clinical score comparable to CFA mice. EAMG mouse model presents the advantage of having a high production of anti-TAChR antibodies which effects are easily observed. Thus, since our treatment reduced inflammation and activation of B cell in the spleen of mice, we were able to observe an important reduction in antibody production that was reflected in an amelioration of clinical symptoms.

Of note, we only test one posology (100µg/mouse/week/4 weeks). Therefore, it should be considered to test different concentrations as reported in other pre-clinical models [70-72].

CONCLUSION

Our results highlight the critical role of Th17 cells in the development of Myasthenia Gravis. We showed that a therapy with monoclonal anti-IL-23p19 is effective to decrease the inflammation in MG thymus and control the formation of eGC which is a hallmark of $AChR^+$ MG thymus. More, we corroborate in EAMG mouse model our observations and showed that blocking IL-23p19 reduces the production of IL-17 and the production of antibodies. This engenders an amelioration of clinical symptoms and an improvement in muscle physiology. Therefore, we show for the first time the potential therapeutic effects of a monoclonal anti-IL-23p19 antibody in the physiopathology of Myasthenia Gravis.

CONFLICT OF INTEREST

The authors declare that they have no relevant conflict of interest.

AUTHORS CONTRIBUTIONS

J.V., J.V.W., J.M. and K.M performed the experiments, analyzed the data and interpreted the results. F.T. provided help to obtain samples. K.M, C.F and P.P performed the ELISA experiments. R.R. and N.S provided human thymic tissues. R.L.P. provided helpful suggestions to design experiments. S.B-A. initiated the study. N.D. and S.B-A were involved in all aspects of the study including design, data analysis and interpretation of the results. J.V. and N.D. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Table 1. Characteristics of MG patients included in NSG-MG experiments

MG THYMUS	**AGE (years)**	**MG SCORE** (MGFA)	**STADE**	**GERMINAL CENTERS**	**ANTI-AChR (nmol/L)**	**TREATMENT**
MG # 1	23	18/50	3A/2A	N/A	5.8	Prednisone Pyridostigmine
MG # 2	26	90/100	N/A	Some	>100	Without corticosteroids
MG # 3	26	N/A.	N/A	Some	>100	Without corticosteroids, IVIg
MG # 4	22	74/100	N/A	Some	3.3	Prednisone, Pyridostigmine, Azathioprine, IVIg

Table 2. List of primers used in the study

GENE		Forward	Reverse
HUMAN	**AID**	AAGGGCTGCATGAAAATTCAGT	CGTCTCGTAAGTCATCAACCTC
	BLIMP1	AAGCAACTGGATGCGCTATGT	GGGATGGGCTTAATGGTGTAGAA
	CXCL13	CTCTGCTTCTCATGCTGCTG	TGAGGGTCCACACACACAAT
	GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG
	IFN-γ	TCCCATGGGTTGTGTGTTTA	AAGCACCAGGCATGAAATCT
	IL-17A	CCCCTAGACTCAGGCTTCCT	AGTTCATTCTGCCCCATCAG
	IL-6	TGAGGTGCCCATGCTACATTT	TCTGCGCAGCTTTAAGGAGTT
	KERATIN 14	TTCTGAACGAGATGCGTGAC	GCAGCTCAATCTCCAGGTTC
	KI67	AAGCCCTCCAGCTCCTAGTC	TCCGAAGCACCACTTCTTCT
	PODOPLANIN	TGTGGCGCTTGGACTTTGT	GTGTAACAGGCATTCGCATCG
	ST6GAL1	TGCAGCCTCACGACAGATAC	ACCCTGAGAGACCTTCAGCA
MOUSE	***Aid***	CCAGACTTTGGGTCGTGAAT	TGGCTTGTGATTGCTCAGAC
	Cypa	CACCGTGTTCTTCGACATCAC	CCAGTGCTCAGAGCTCGAAAG
	Gapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
	Il-17a	TCTCTGATGTTGCTGCT	CGTGGAACGGTTGAGGTAGT
	Il-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
	Il-6R	GACTATTTATGCTCCCTGAATGATCA	ACTCACAGATGGCGTTGACAAG
	MyoD	AAGACGACTCTGACGGCTTG	TCTGGTGAGTCGAAACACGG
	MyoG	GGGCAAACTCAGGAGCTTCT	CAGAGGCTTTGGAACCGGAT
	Pax7	GGGCTCTTCAAGGTCTGGAC	CAGGGAGCAAGGAATGTGGA
	Podoplanin	GCCAGTGTTGTTCTGGGTTT	AGAGGTGCCTTGCCAGTAGA
	TGF-β1	CAAGGGCTACCATGCCAACT	CCGGGTTGTGTTGGTTGTAGA

Table 3. List of antibodies for flow cytometry and immunohistochemistry analyses

Antibody	Conjugate	Host	Reactivity	Clone	Supplier
CCR4	PE	Mouse	Human	205410	R&D
CCR6	APC	Mouse	Human	11A9	BD Bioscience
CD127	FITC	Mouse	Human	ebioRDR5	ebioscience
CD19	FITC	Mouse	Human	HIB19	ebioscience
CD25	PE	Mouse	Human	BC96	ebioscience
CD4	V450	Mouse	Human	RPA-T4	BD Bioscience
CD45	V500	Mouse	Human	H130	BD Bioscience
CD8	ACP-H7	Mouse	Human	SK1	BD Bioscience
IL-23R	PerCP	Mouse	Human	218213	R&D Systems
Keratin 14	Purified	Rabbit	Human	Poly19053	Biolegend
Ki-67	Purified	Rat	Human	5D7	Abcam
Lamin A/C	Purified	Mouse	Human	636	Leica
CCR6	APC	Rat	Mouse	140706	BD Pharmingen
CD127	V450	Rat	Mouse	SB199	BD Bioscience
CD25	FITC	Rat	Mouse	16184	BD Bioscience
CD31	Purified	Rat	Mouse	MEC 13.3	BD Pharmingen
CD4	APC-eF780	Rat	Mouse	GK 1.5	ebioscience
CD62L	PerCP Cy5.5	Rat	Mouse	MEL14	ebioscience
CD8	PE Cy7	Rat	Mouse	53-6.7	BD Bioscience

FIGURE LEGENDS

Figure 1. Anti-IL-23p19 treatment does not induce global physiological changes in NSG-MG mouse model. Representative images of vascularized human MG thymus after engraftment in mice without **A)** or with treatment **B)**. Flow cytometry analyses of human $CD4^+$sp cells **C)**, $CD8^+$sp cells **D)** and $CD19^+$ cells **E)** in engrafted human MG thymuses in NSG mice. ELISA quantification of total human immunoglobulins in serum of NSG-MG mice **F)**. Flow cytometry analysis of human $CD4^+$ cells **G)** and $CD19^+$cells **H)** in blood of NSG-MG mice. Spleen weight of engrafted NSG-MG mice **I)**. Flow cytometry analysis of human $CD4^+$ cells **J)** and $CD19^+$ cells **K)** in spleen of NSG-MG mice. mRNA expression of human Ki-67 in spleen of engrafted NSG-MG mice **L).** Representative images of NSG-MG spleen stained with anti-human Lamin A/C (green) and anti-human Ki67 (red) with **M)** or without treatment **N)**. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope using 20× magnification. In flow cytometry analyses, each point represents the percentage of cells per mouse from 4 different experiments. All analyses were done at day 42 after engraftment in NSG-MG mice treating with saline solution (NaCl) or anti-IL-23p19. The data are from 4 different experiments done with 4 thymic biopsies obtained from 4 different MG patients. For each thymic biopsy, n>3 mice per treatment condition. mRNA expressions were done in duplicate and analyzed by real-time PCR. mRNA expressions were expressed as arbitrary unit (AU) and normalized to *GAPDH*. P values were obtained using non-parametric Mann-Whitney test.

Figure 2. Anti-IL-23p19 treatment reduces level of Th17 cell pathogenic markers within $AChR^+$ MG human thymuses

Protein analyses of *IL-6* **A)**, *TGF-β1* **B)**, *IL-21* **C)**, *TGF-β3* **D)**, *IL-17A* **E)** from global human $AChR^+$ MG thymic biopsies engrafted in NSG-MG mice. Flow cytometry analyses of $CCR6^+CCR4^+$ T-cells **F)** and $CCR6^+IL\text{-}23R^+$ T-cells **G)** among $CD4^+$sp T-cells in engrafted thymic biopsies. Protein analysis of IL-2 from global human $AChR^+$ MG thymic biopsies engrafted in NSG-MG mice **H)**. Flow cytometry analysis of $CD25^+CD127^-$ T-cells among $CD4^+$sp T-cells in engrafted thymic biopsies **I)**. mRNA expression of *IFN-γ* in global human thymic biopsies **J)**.

All analyses were done at day 42 after thymic engraftment in NSG-MG mice treated with saline solution (NaCl) or anti-IL-23p19. All the data are from 4 different experiments done with 4 thymic biopsies obtained from 4 different MG patients. For each thymic biopsy, n>3 mice per treatment condition. Protein analysis was done by ELISA and each point represents the mean value of at least 3 mice engrafted with thymic biopsies obtained from one donor. For flow cytometry and RT-PCR analyses, each point represents one mouse from at least 4 different engrafted thymuses. mRNA expressions were analyzed by real-time PCR and are expressed as arbitrary unit (AU) and are normalized to *GAPDH*. P values for ELISA were obtained using one-tail Wilcoxon matched paired test. P values for cytometry and RT-PCR analysis were obtained with Mann-Whitney test.

Figure 3. Effects of anti-IL-23 treatment on B cell-associated proteins and thymic stromal cells in AChR$^+$ MG thymuses

mRNA expression levels of *AID* ***A)***, *PODOPLANIN* ***B)***, *CXCL13* ***C)***, *BLIMP1* ***D)***, *ST6GAL1* **E)** and *KERATIN 14* **F)** in AChR$^+$ MG thymic engrafted in NSG-MG mice. Representative images of stromal cells stained with anti-human Keratin 14 (green) AChR$^+$ MG thymic biopsies engrafted in NSG-MG mice without **G)** or with treatment **H)**. All analyses were done at day 42 after thymic engraftment in NSG-MG mice treating with saline solution (NaCl) or anti-IL-23p19. All the data are from 4 different experiments done with 4 thymic biopsies obtained from 4 different MG patients. For each thymic biopsy, n>3 mice per treatment condition. mRNA expressions were analyzed by real-time PCR and are expressed as arbitrary unit (AU) and normalized to *GAPDH*. P values were obtained with Mann-Whitney test.

Figure 4. Effect of anti-IL23 treatment on human T cells and associated cytokines in blood and spleen of engrafted NSG mice

Analysis by flow cytometry of human CCR6$^+$ CCR4$^+$ cells **A)**, CCR6$^+$ IL-23R$^+$cells **B)** and CD25$^+$CD127$^-$cells **C)** in blood of NSG-MG mouse. Analysis by flow cytometry of human

$CCR6^+$ $CCR4^+$ cells **D)**, $CCR6^+$ IL-$23R^+$ cells **E)** and $CD25^+$ $CD127^-$ cells **F)** in NSG-MG mouse spleen. ELISA analysis of human anti-AChR antibodies in blood of NSG-MG mice 28 days after thymic engraftment **G)**.

Flow cytometry analyses were done at day 42 after thymic engraftment in NSG-MG mice treating with saline solution (NaCl) or anti-IL-23p19. All the data are from 4 different experiments done with 4 thymic biopsies obtained from 4 different MG patients. For each thymic biopsy, n>3 mice per treatment condition. For flow cytometry data, each point represents one mouse from 4 different engrafted thymuses. For ELISA, each point represents the mean value of at least 3 mice engrafted with thymic biopsies obtained from one donor. P values were obtained with Mann-Whitney test (Flow cytometry) and Wilcoxon matched paired test (ELISA).

Figure 5. Anti-IL-23p19 treatment reduces inflammation status in EAMG mouse blood

Analysis of serum level of IL-17A in EAMG mice by ELISA **A)**. Flow cytometry analysis of the percentage of circulating $CD4^+CD62L^-CCR6^+$ cells **B)** and $CD4^+CD25^+CD127^-$ cells **C)**. ELISA analyses were performed in duplicate with samples (day 49 after immunization) obtained from two independent experiments. Flow cytometry data are representative treatment data of one representative experiment. n>3 mice per group and per treatment for each experiment. P values were obtained with Mann-Whitney test.

Figure 6. Anti-IL-23p19 treatment reduces the serum concentration of antibodies anti T-AChR

ELISA analysis of the serum level of total anti-T-AChR antibodies **A)**, anti-T-AChR antibody IgG1 subtype **B)** and anti T-AChR antibodies IgG2b subtype **C)** in CFA and EAMG mice. Anti-T-AChR antibodies of subtypes IgG1 and IgG2 were normalized on the total IgG level. Analyses were performed in duplicate on serum obtained at day 49 after initial immunization. Data are obtained from 2 independent experiments. n>3 mice per group and

per treatment for each experiment. Each point represents an individual mouse. P values were obtained with Mann-Whitney test.

Figure 7. Anti-IL-23p19 treatment decreases the activation of satellite cells and the inflammation status in EAMG muscle

mRNA expression analysis of *Pax7* **A)**, *MyoD* **B)**, *MyoG* **C)**, *Tgf-β1* **D)**, *Il-6* **E)**, *Il-6r* **F)** and *IL-17a* ***G)*** in muscle of CFA and EAMG mice. Analyses were done at day 49 after immunization. Data are obtained from one representative experiment. n≥4 mice per group and per treatment. Each point represents an individual mouse. mRNA expressions were done in duplicate by quantitative RT-PCR. mRNA are expressed as arbitrary unit (AU) and normalized to *Cypa.* P values were obtained with Mann-Whitney test.

Figure 8. Effects of anti-IL-23p19 treatment on myasthenic clinical symptoms in EAMG and NSG-MG models

Weekly analysis of global clinical score in NSG-MG mice **A)** and EAMG mice **B)**. Data for NSG-MG (n≥11 per group) and EAMG (n≥14 per group) mice were obtained from 3 independent experiments. NSG-MG model was done with 3 human thymic biopsies obtained from 3 different MG patients. For each thymic biopsy, n> 4 mice per treatment condition. P values were obtained with t test.

Supplementary Figure 1 Anti IL-23p19 tends to reduce markers of eGC in spleen of EAMG mice

mRNA expression of *Podoplanin* **A)** and *Aid* **B)** in spleen of CFA and EAMG mice with or without anti-IL-23p19. Analyses were done at day 49 after initial immunization. Data are obtained from 2 different experiments. n>4 mice per group and per treatment for each experiment. Each point represents an individual mouse. mRNA expressions were done in duplicate by quantitative RT-PCR. mRNA are expressed as arbitrary unit (AU) and normalized to *Gapdh.* P values were obtained with Mann-Whitney test.

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RESULTS

Figure 1. Anti-IL-23 treatment does not induce global physiological changes in NSG-MG mouse model.

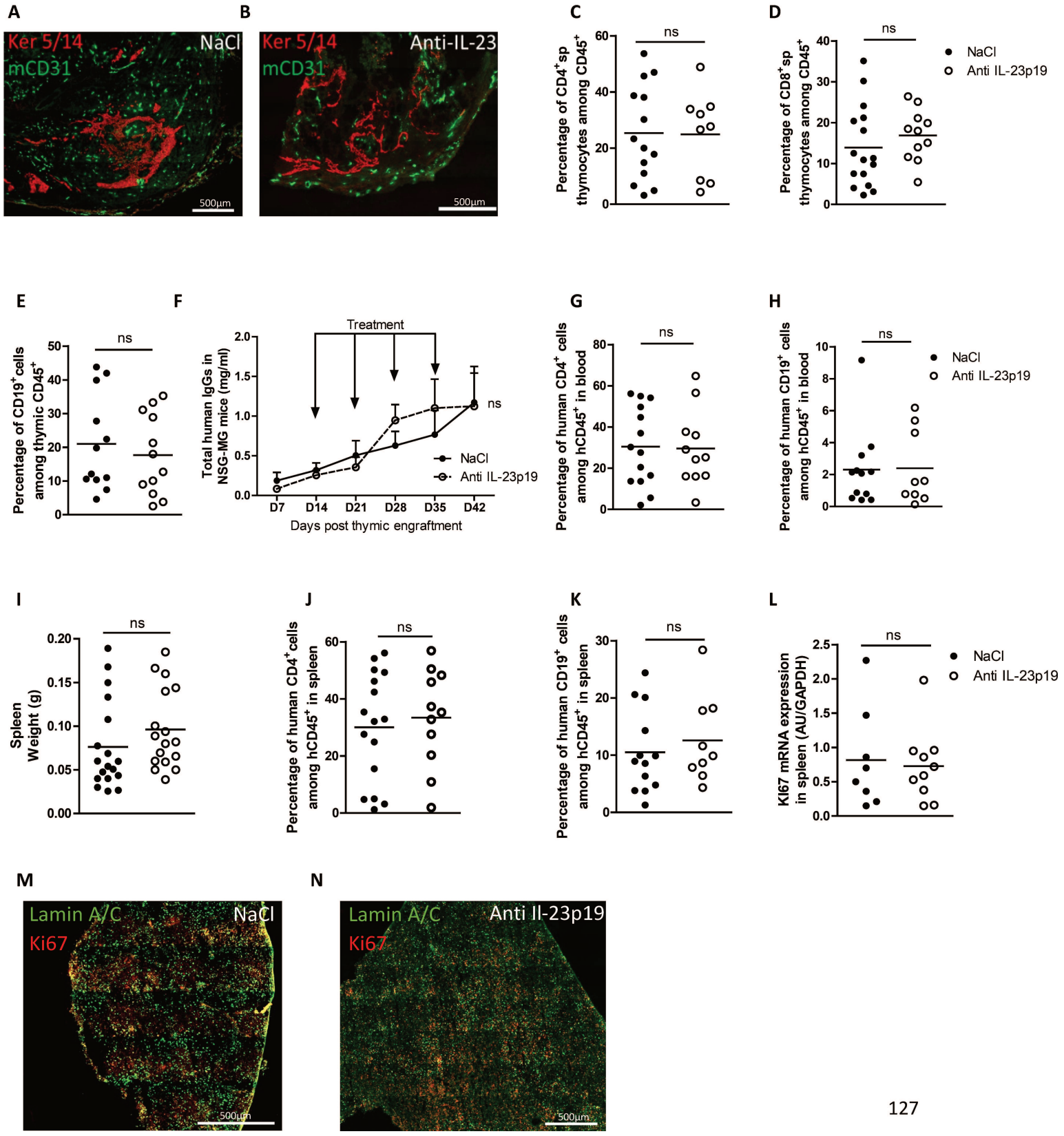


Figure 2. Anti-IL-23p19 treatment reduces level of Th17 cell pathogenic markers within AChR$^+$ MG human thymuses

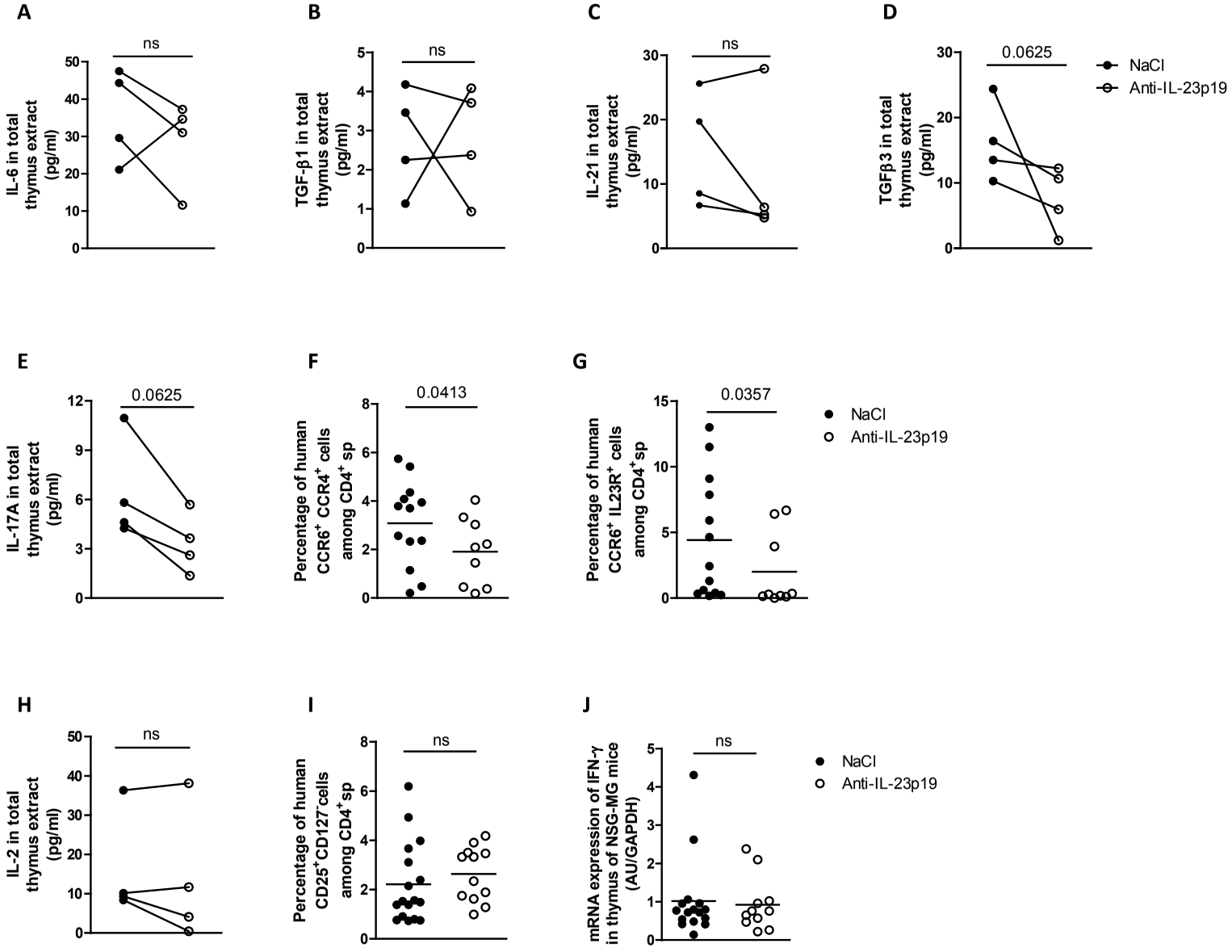


Figure 3. Effects of anti-IL-23 treatment on B cell-associated proteins and thymic stromal cells in AChR^{+} MG thymuses

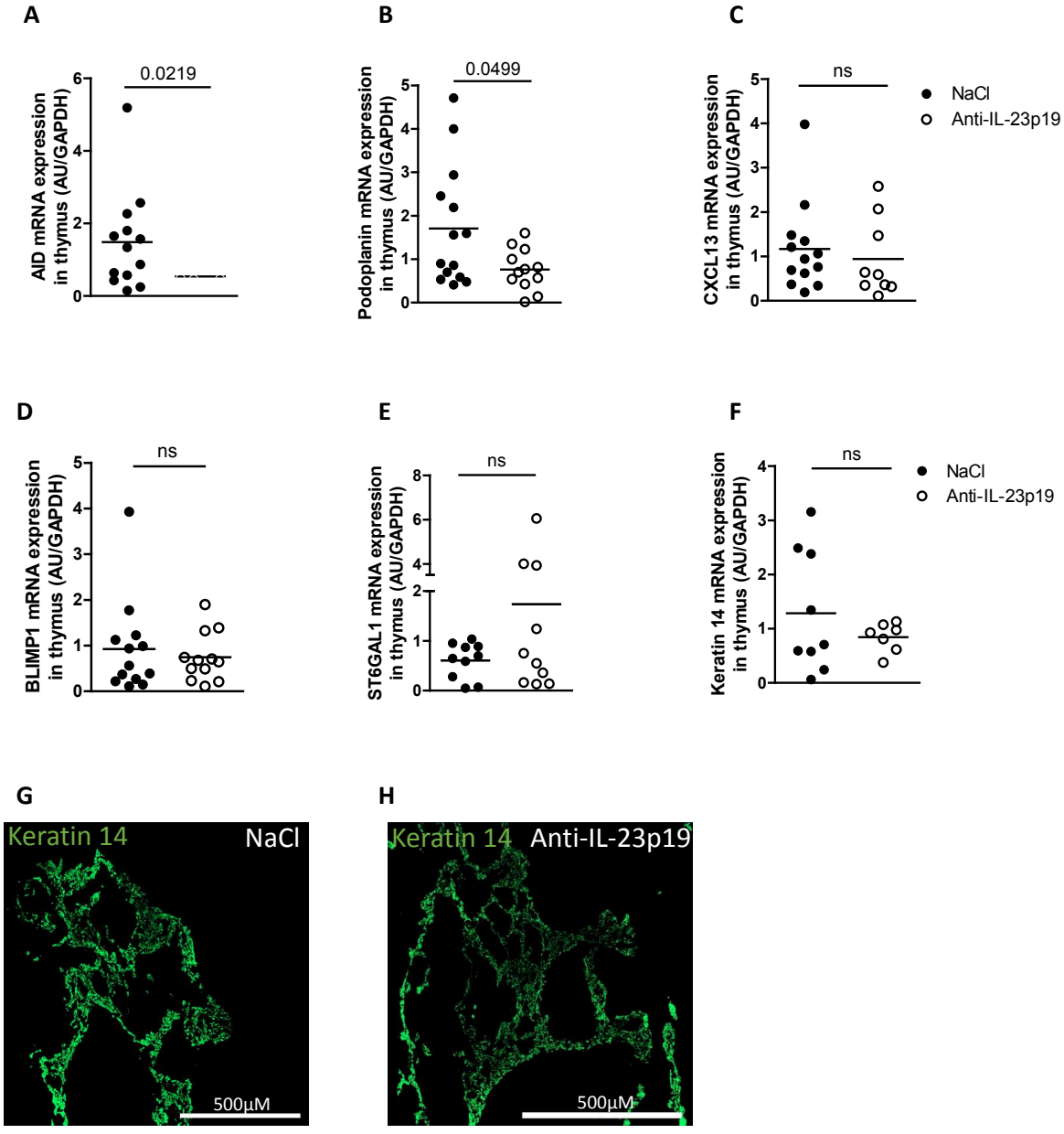


Figure 4. Effect of anti-IL23p19 treatment on human T cells and antibodies in blood and spleen of engrafted NSG mice

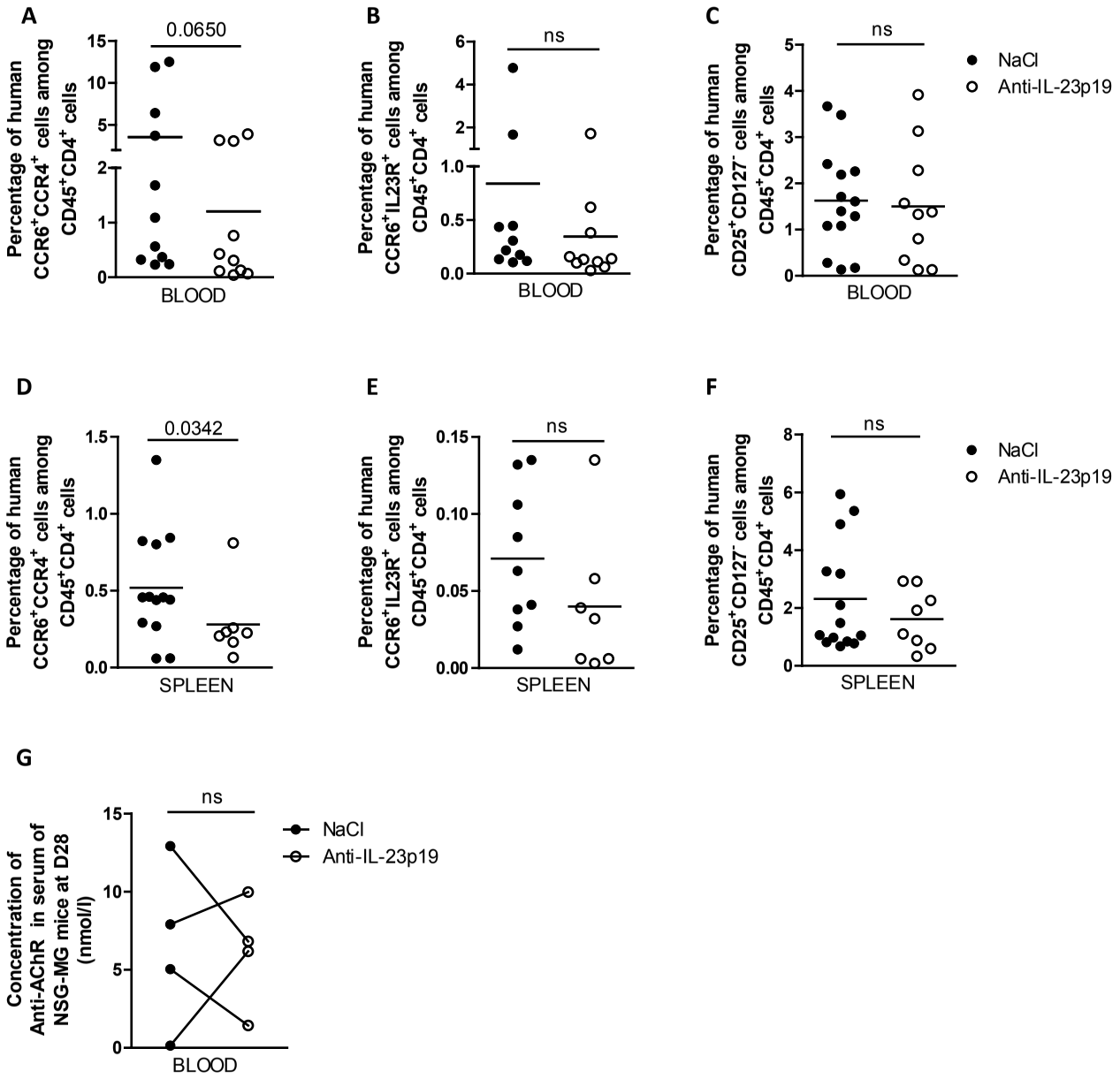


Figure 5. Anti-IL-23p19 treatment reduces inflammation status in EAMG mouse blood

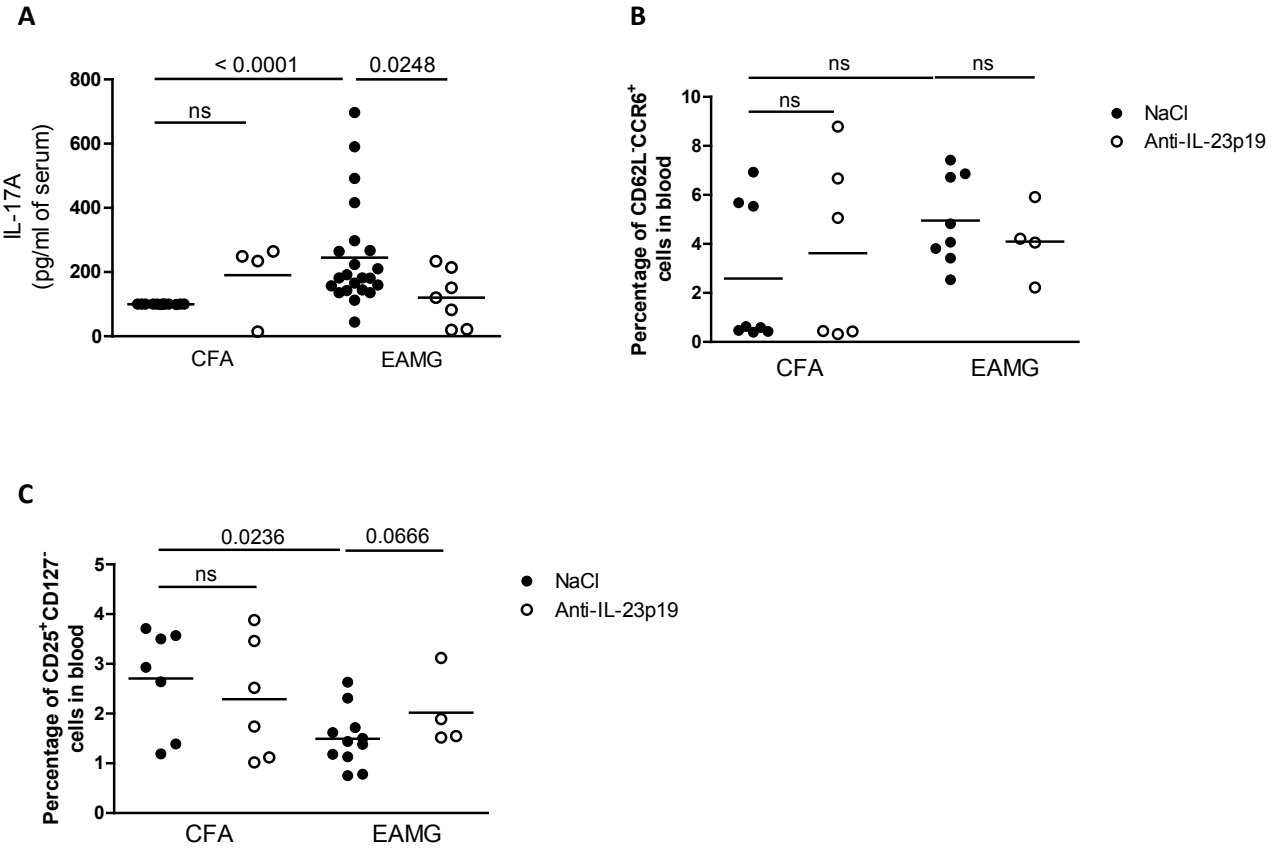


Figure 6. Anti-IL-23p19 treatment reduces the serum concentration of anti T-AChR antibodies

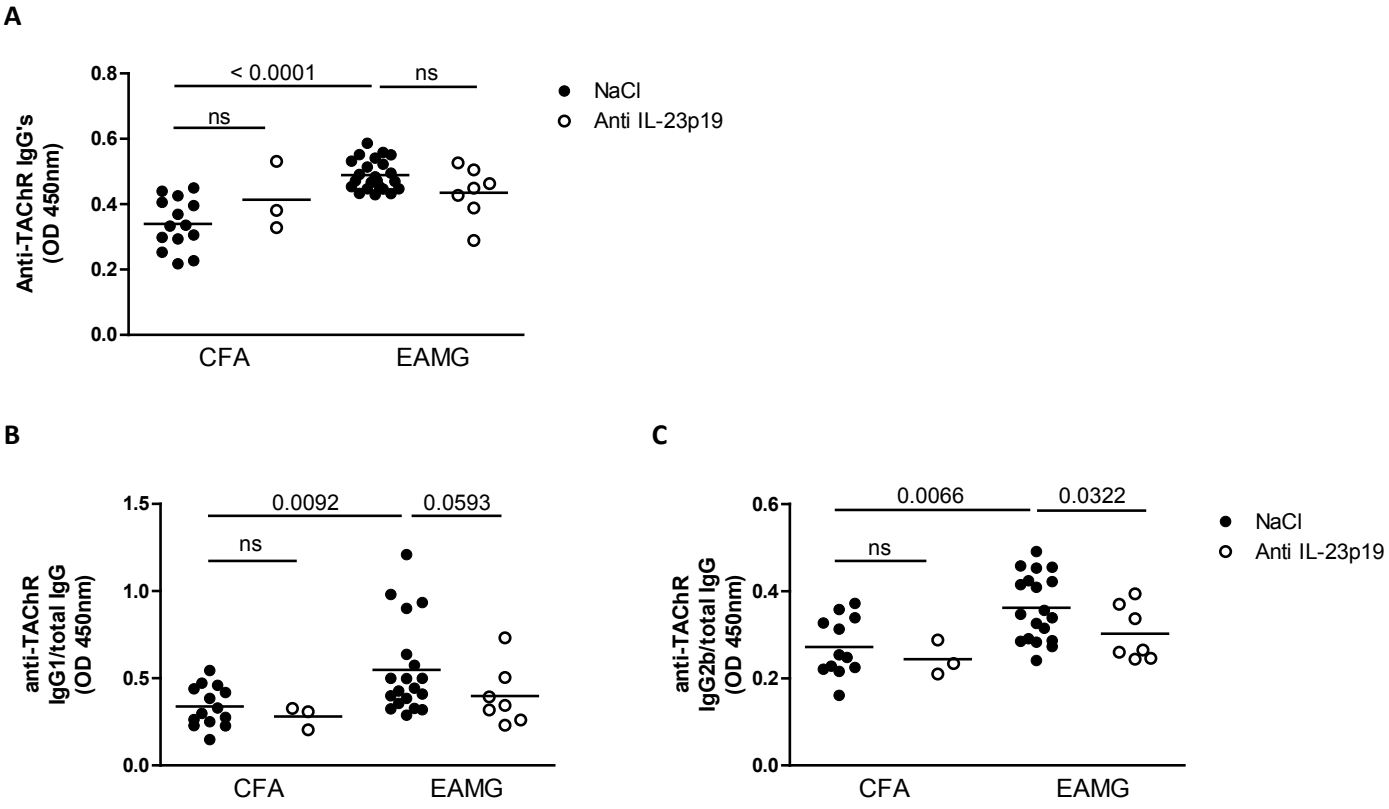


Figure 7. Anti-IL-23p19 treatment decreases the activation of satellite cells and the inflammation status in EAMG muscle

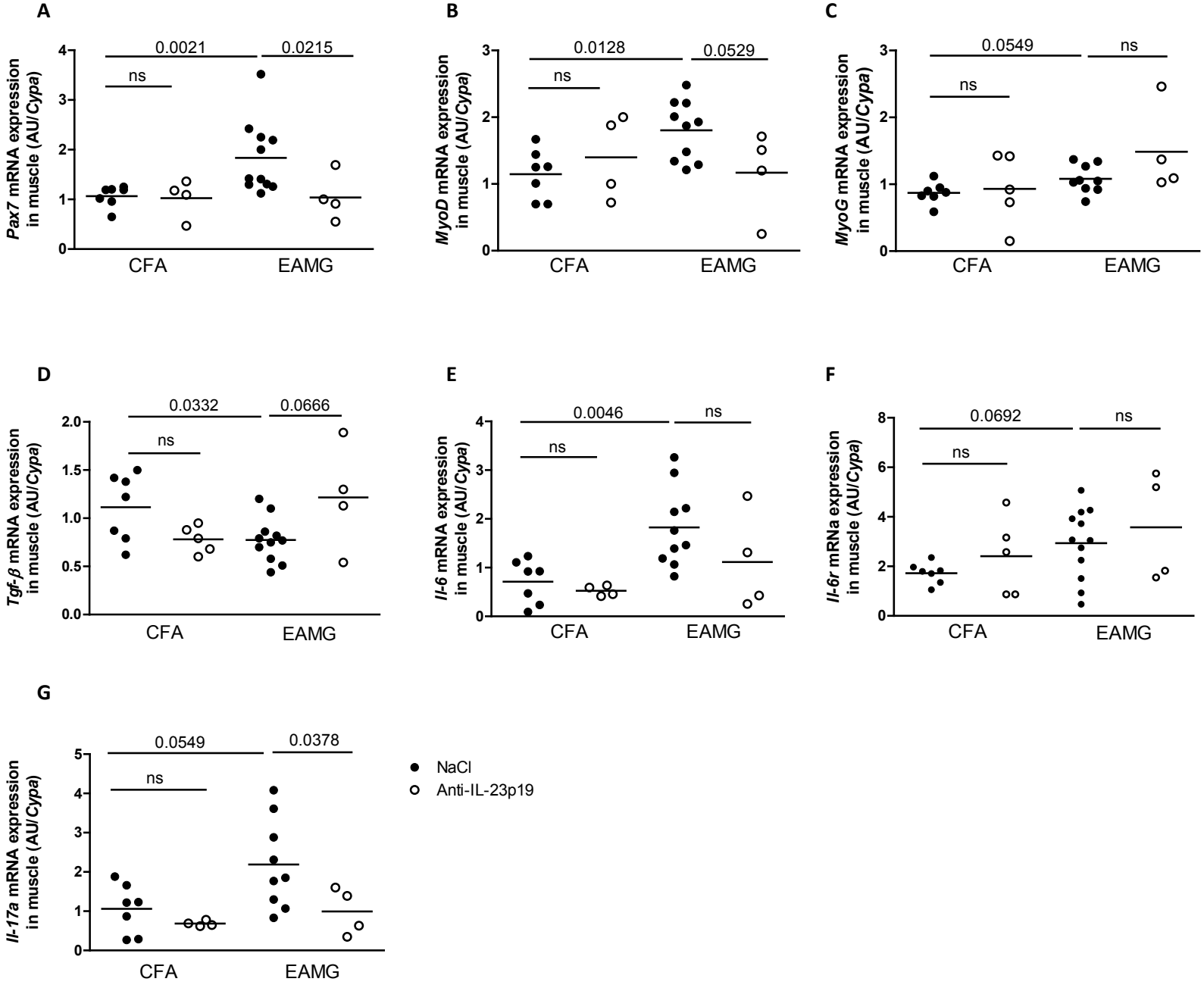
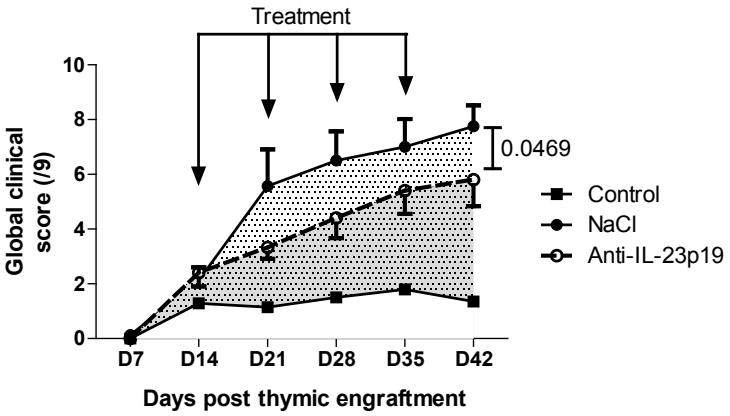


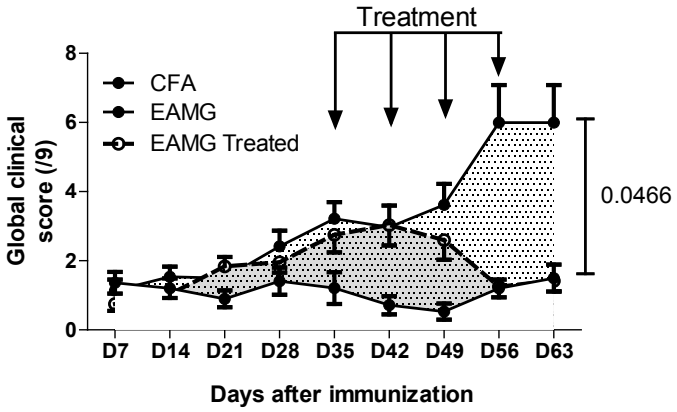
Figure 8. Effects of anti-IL-23p19 treatment on myasthenic clinical symptoms in NSG-MG and EAMG models

A



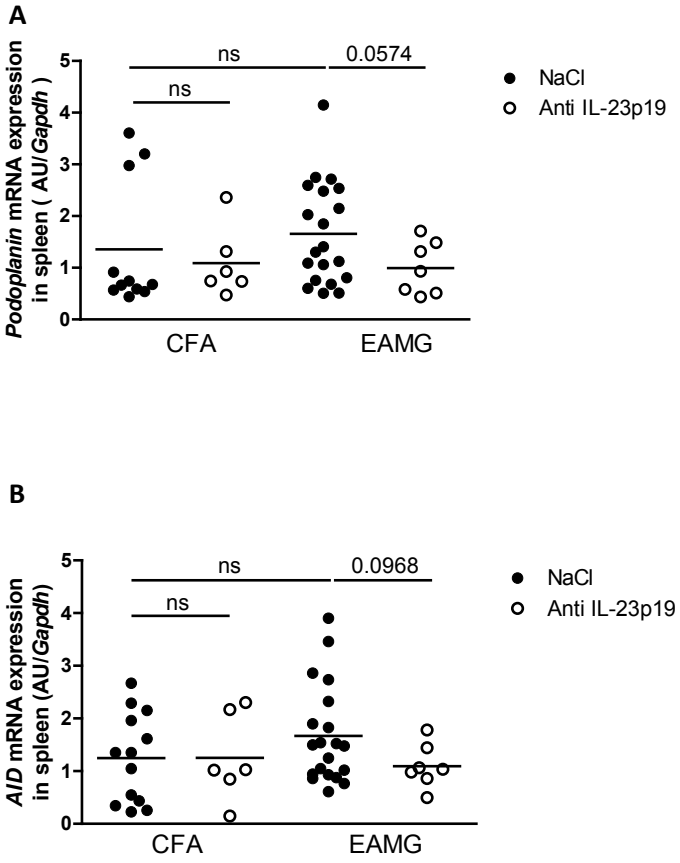
	NSG-MG	NSG-MG treated
Mean	25.69	17.56
Std. Deviation	9.254	5.955
Std. Error	3.272	1.985

B



	TAChR	TAChR treated
Mean	19.94	13.01
Std. Deviation	10.91	6.011
Std. Error	2.915	1.503

Supplementary Figure 1 Anti IL-23p19 tends to reduce markers of eGC in spleen of EAMG mice



III. DISCUSSION AND CONCLUSIONS

The objective of this work was to search for a new therapeutic approach to treat Myasthenia Gravis. To date, Myasthenia gravis treatments are based on anticholinesterases, glucocorticoids and immunosuppressors [450]. Even if these therapies are useful to control some symptoms of the pathology, they do not provide a cure and may come with several side effects. A clinical trial has shown that thymectomy is effective to reduce clinical symptoms in AChR$^+$ MG patients with a disease duration less than 5 years, and high level of AChR antibodies. However, patients still needed to be treated with glucocorticoids although a lower dose [410, 461]. Plasma exchange and IVIg are two therapeutic approaches that remain useful to treat myasthenic crisis but they do not aim neither to control the immune system and the dysfunctional thymus [456]. Therefore, to look for new therapies in myasthenia gravis that control the immune dysregulation and the disease effector tissue, the thymus, and consequently reduce the clinical symptoms, is still needed.

Does the IL-23/Th17 cells pathway is involved in MG?

Most autoimmune diseases including myasthenia gravis display chronic inflammatory process that increases production of cytokines that promote the development of Th17 cells [4, 36, 132, 195]. Among these cytokines, IL-23 is the “master cytokine” that changes the fate of Th17 cells and promotes a pathogenic phenotype sustaining autoimmune diseases development. The use of antibodies targeting the IL-23/Th17 pathway has emerged and has provided interested results for pathologies like psoriasis, RA and Crohn’s disease [124, 125, 466].

Few but strong data have suggested the involvement of IL-23/Th17 pathway in MG pathogenesis in the thymus and in the periphery. For instance, a report showed that AChR$^+$ MG thymus displayed an increased expression of IL-17 in regulatory and effector T-cells [417]. More, the increased level of Th17 related cytokines such as IL-1β, TGF-β1 and IL-6 in MG thymuses have been demonstrated [93, 418, 419]. During my doctoral work, I add another piece of to the puzzle by demonstrating that IL-23 is also increased in MG thymus. Indeed, MG TEC and not DC overexpressed IL-23 consequently to the highly dysregulated IFN-I pathway [329]. This observation is in line with what has been observed in systemic lupus erythematosus where the IFN-I pathway is active and has been correlated with an increased number of IL-17 expressing CD4$^+$ cells [467]. More, the activation of IFN-I pathway following TLR3 activation, has been shown to induce specifically, in vitro, the expression of α-AChR by mTEC [324]. Therefore, it is possible that the inflammatory status of the thymus might modified mTEC physiology, promotes the expression of pro-inflammatory cytokines and then modulates T-cell differentiation (favoring a Th17 phenotype) but also thymocyte selection

(through a TSA biased presentation). In addition, IL-23 has been proposed to enhance antigen presentation by DC in the skin and in allergic processes [116, 468] but whether MG thymic DC are stimulated by IL-23 remains to be determined. It is worth mentioning that, mTEC are also responsive to IL-17 by increasing the expression of IL-23 which probably amplifies Th17 related inflammation in MG thymus.

Moreover, in addition to IL-23, pathogenicity of Th17 cells can also be controlled by other molecules such as serum/glucocorticoid-regulated kinase 1 (SGK1), CD5 antigen-like (CD5L) and receptor interacting protein 2 (RIP2) sensors of NaCl, lipids and a signaling adaptor protein for intracellular receptors that recognize peptidoglycans [78, 79, 84, 87]. Such factors may brought additional information on MG patients, and drawn the picture of environmental features that may participate to disease physiology as observed in the mouse model of MS [76] and the different clinical studies rheumatoid arthritis [469, 470]. Of these regulators of pathogenicity, I analyzed the expression of RIP2 and did not observed significant difference between control and AChR$^+$ MG patients (data not shown). However, our analyses were done in total thymocytes and subtle changes that affected Th17 cells should have been done on sorted thymic Th17 cells to obtain conclusive data. In the same line, a deeper analysis is required to determine whether MG thymic Th17 cells could be impacted by CD5L and SGK1 activations, indicating that the various factors cross talk to control the pathogenicity of these cells in MG thymuses.

During my doctoral work, I have confirmed the presence of CD4$^+$ cells expressing IL-17 in the AChR$^+$ MG thymus and I have identified thymic CD4$^+$ cells expressing podoplanin around eGC. Podoplanin is an anchoring protein that is highly expressed by Th17 cells [201]. In mouse models of MS and RA, Pdpn$^+$ Th17 cells have been linked to the formation of eGC, and high expression of IL-17 [201, 205]. However, so far, the role of Pdpn in human Th17 cells is not clearly elucidate. For instance, a report showed that Th17 cell differentiated in the presence of IL-6, IL-1β, TGF-β1 and IL-23 induced the expression of Pdpn by Th17 cells. But, these cells do not secrete neither IL-17 nor IFN-γ [203]. In contrast, another report has shown that PBMC cultured in contact with synoviocytes of RA patients induced a high expression of IL-17 with a critical role of Pdpn in this IL-17 production [204]. Therefore, in line with these different observations, one can imagine that MG thymic Th17 cells may contribute to the stabilization of eGC, structures that are highly involved in the production of autoantibodies that attack the AChR proteins. We could then envisage that thymic pathogenic Th17 cells might play a role in the antibodies induced the development of clinical symptoms. But, a

singular feature of AChR$^+$ MG is the lack of correlation between the antibody concentrations and the clinical signs of the patients [267]. However, this apparent dichotomy may be explained by the fact that IL-23 induced Th17 cell controls, in addition to the antibody production [201, 218], the autoantibodies pathogenicity in RA [154]. Indeed, IL-21 and IL-22 productions by Th17 cells can affect the expression of ST6gal1, an enzyme present in B-cells that controls the sialylation of IgG and thus controls IgG pathogenicity [154]. In AChR$^+$ MG thymuses, IL-21 is overexpressed [417] and ST6gal1 display a reduced expression (data not published). Therefore, thymic Th17 cells in MG patients may contribute to regulate in B-cells expression of ST6gal1 and modulate the AChR$^+$ IgG pathogenicity. Nevertheless, Selman et al have previously used a MG cohort that encounters diverse MG patients profiles (young and older) and have reported no gross differences for the level of IgG1 and IgG2 Fc sialylation but at the individual IgG glycoform level, and a higher prevalence of specific modification in IgG1 and IgG2 species for AChR MG patients compared to the controls [471]. Therefore, more investigations remain to be done to validate or to invalidate this hypothesis.

Moreover, chemokines also play an important role the pathogenicity process mediated by thymus in MG. Indeed, B-cell are chemoattracted to the thymus by CXCL13 and CCL21, chemokines overexpressed in MG thymus [403, 404]. More, MG thymus present as well an abnormal angiogenic process with the development newly formed high endothelial venules (HEV) [472]. In MG thymus, HEV were shown to express CXCL12 another chemokine implicated in B-cell attraction but also of myeloid and plasmacytoid DC expressing CXCR4 [472]. Interestingly, mice injected with Poly(I:C), a molecule know to activate IFN-I pathway, displayed symptoms of MG such as a reduced grip strength, presence of anti-AChR antibodies and a reduction of AChR. In addition, this mouse model showed a thymic overexpression of CCL21 and CXCL13 [324]. More, epithelial cell are known to overexpress chemokines including CCL20 after IL-17 stimulation [126, 133]. Here, I showed an increased number of CD4$^+$CCR6$^+$ cells, CCL20 being the chemokine ligand of CCR6. Then, in addition to the overexpression of B-cell chemoattractant [403, 404], MG thymus could also present an increased production of Th17 cell chemoattractant CCL20, however this remains to be explored.

Altogether, **figure 18** illustrates the results that I have obtained in addition to the literature, highlighting a potential loop of inflammation occurring in MG thymus. In this mechanism, an activation of IFN-I pathway may trigger the expression of pro-inflammatory factors including IL-23. Then, IL-23 enhances the development of IL-17 producing cells that on one side induces and sustains

the formation of eGC and on the other side re-activates mTEC to produce IL-23. This creates a continuous loop of inflammation between Th17 cells and mTEC.

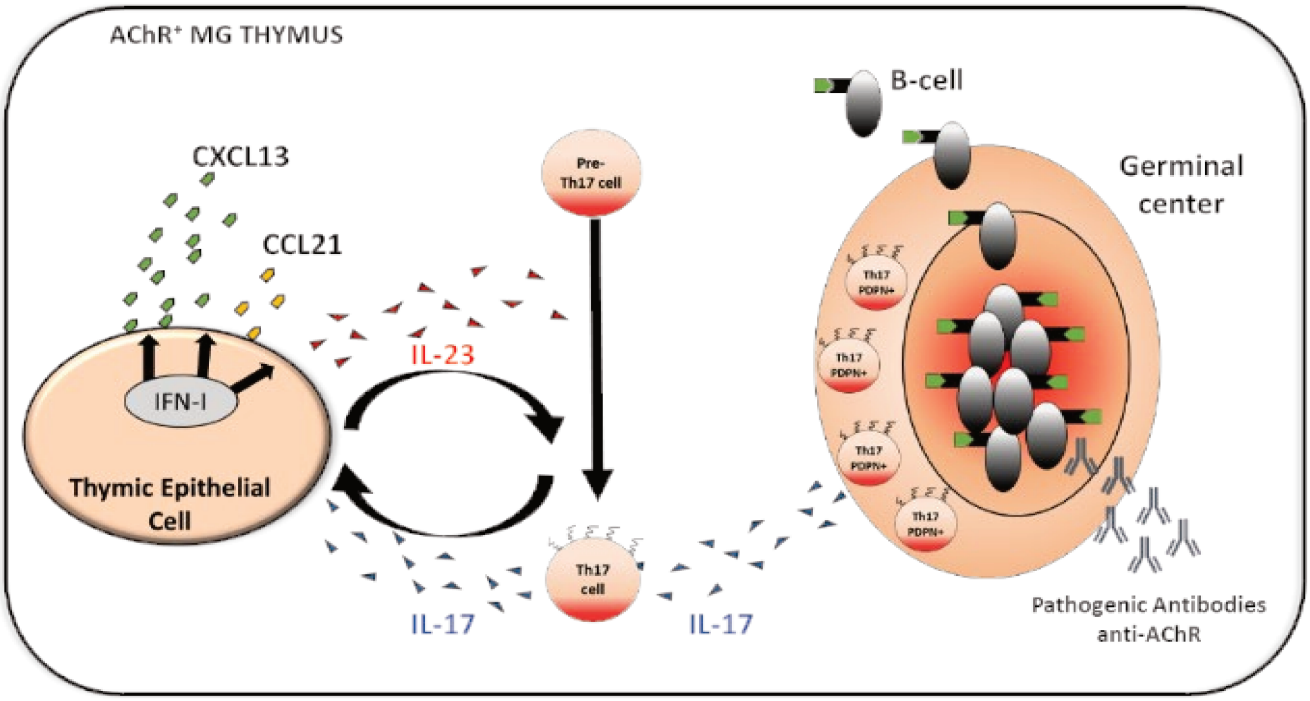


Figure 18: Schematic representation of IL-23/IL-17 loop of regulation in AChR+ MG thymuses

Therefore, these data lead us to identify a new therapeutic target to control the inflammation in the thymus and in the periphery.

Of note, targeting the IL-23/Th17 cell pathway with monoclonal antibodies has been widely used to calm inflammation and provided optimistic results for pathologies such as Crohns disease, psoriasis and SLE [124, 125, 236].

Targeting the IL-23/Th17 cell pathway in preclinical models of MG

Since my first doctoral results have shown that IL-23 to be a critical player in the development of MG thymic inflammation, we evaluate the effects of an anti-IL-23p19 monoclonal antibody on the clinical symptoms and on the thymic inflammation and homeostasis.

Various pre-clinical mouse models have described numerous therapeutic strategies with doses of monoclonal anti-IL-23 used, ranged from 20µg/mouse/6 weeks to two injections with 1mg/mouse [473-476]. In most of these pre-clinical experiments, mice are treated before the development of the pathology, in order to assess the capacity of anti-IL23 to limit the pathology development [475, 476] but not to ameliorate an established pathology. By contrast, in the context of psoriasis, a clinical trial have investigated the impact of the therapy on disease affected patients. This clinical trial showed that patients receiving anti-Il-23p19 with doses ranging from 10mg to 300mg in a single dose achieved a significant disease amelioration [477]. Therefore, based on these reports, we initiate a treatment with a weekly injection of 100µg of anti-IL-23p19. Following the published recommendations [478], clinical tests and biological analysis (blood) were done double blinded to avoid any bias.

EAMG is the most widely used mouse model for AChR^{+} MG. By using this model, some reports have corroborated the potential influence of Th17 cells in EAMG development (**Figure 19)**.

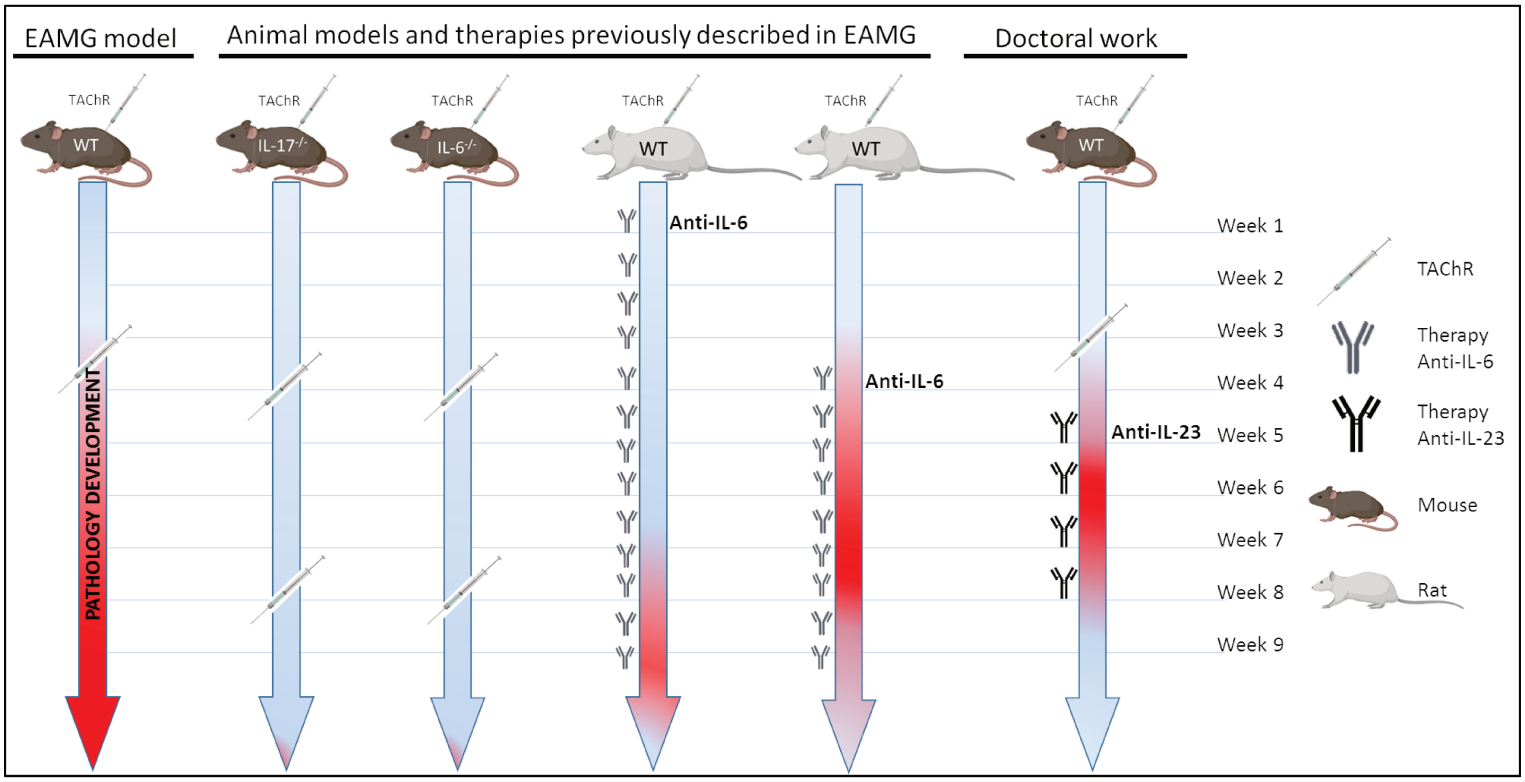


Figure 19: Th17 cells in the development of EAMG.

For instance, deficiency of IL-17 or IL-6 protects mice for the induction of EAMG, reduced the production of anti-TAChR antibodies and the germinal centers in the spleen [479, 480]. These reports show evidence of the critical roles of cytokines involved in the development of Th17 cells such as IL-6 as well as a protein produced by th17 cells (IL-17) in EAMG induction. Furthermore, Aricha et. al. showed that treating EAMG rats with an anti-IL-6 monoclonal antibody reduced clinical signs of fatigue [94]. Interestingly, a treatment with anti-IL6 in an acute phase (1 week after induction) of EAMG development inhibited in 75% of rats the apparition of clinical symptoms. The anti-IL-6 treatment contributes to reduce the expression of pro-inflammatory cytokines like, IL-21, IL-17, IL-23 and TNF-α in rats treated in the acute phase [94]. By contrast, targeting IL-6 at the chronic phase (4 weeks after EAMG induction) induced improvement for only 50% of rats.

I showed that treating EAMG mice with anti-IL-23p19 can ameliorate the clinical symptoms and reduce the inflammation after 4 weeks of treatment. Therefore, my results suggest that controlling the development of pathogenic Th17 cell may improve EAMG clinical signs, with a slowdown effect on MG muscle alterations.

Whether EAMG have shown the implication of Th17 cells and their developing cytokines in MG development, one should to keep in mind that this model is an efficient model to analyze the periphery and the muscle alterations. However, EAMG displays an intrinsic limitation since it does not implicate the thymus as the effector tissue. To overcome the EAMG mouse model limitation, in this work, I took advantage of the humanized MG mouse model (NSG-MG mouse model) [449] (**Figure 20**). Since, the objective was to determine the treatment efficiency on sick NSG-MG mice, we carried out the treatment on mice that display obvious chronic disease. $AChR^{+}$ MG clinical symptoms of myasthenia occur 15 days after the engraftment of $AChR^{+}$ MG thymic biopsies in NSG mice [449].

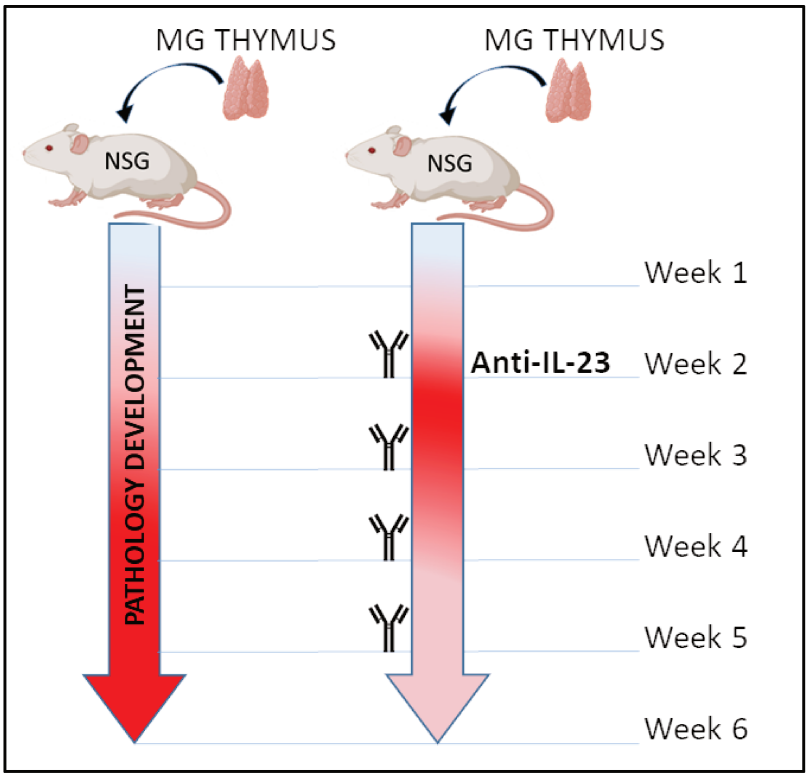


Figure 20: Development of MG in NSG mice engrafted with AChR⁺ MG thymus.

Our results showed that mice engrafted with an AChR⁺ MG thymuses and receiving anti-IL-23p19 antibody had an amelioration of the global clinical score including amelioration of the hanging force and the grip force compared to non-treated mice. This reinforces the data obtained with the EAMG model. More, the amelioration observed in the global clinical score was similar to what have been observed in the EAMG mouse model treated with anti-IL-6 in the chronic phase [94].

IL-23 participate in the pathogenicity of Th17 cells by promoting the endogenous expression of TGF-β3 in Th17 cells [22]. A previous report showed that Th17 cells differentiated in presence of IL-23 and TGF-β3 have a higher potential to induce EAE than Th17 cells differentiated in presence of IL-23 and TGFβ1 [22]. Accordingly, in our NSG-MG model blocking IL-23 reduced the production of TGF-β3 but not TGF-β1. Moreover, IL-23 signaling induces the phosphorylation of STAT3, and enhance RORγt the master transcription factor of Th17 cells [115, 118]. RORγt, as the master transcription factor of Th17 cells, controls the expression of IL-17, CCR6, and IL-23R among others genes [21, 22]. Therefore, my results showed that functional blockade of IL-23 reduced the global concentration of IL-17 in AChR⁺ MG thymus as well as a reduced percentage of CCR6⁺IL-23R⁺ T cells. In addition, Peter

et. al. have shown that Th17 cells differentiated with IL-23 express Pdpn and are present in eGC [201]. In NSG-MG mouse model, we observed a decreased expression of Pdpn and AID in AChR$^+$ MG thymus suggesting an impact on the thymic composition of eGC.

Thymic AChR$^+$ MG Treg cells have been shown to be dysfunctional [415, 417]. In contrast to Th17 cells, Treg cells only require TGF-β1 to develop [481]. Anti-IL-23p19 treatment did not altered neither the concentration of TGF-β1 nor the number of Treg cells in MG thymus while an amelioration of the inflammatory thymic status was observed. Thus, treatment with anti-IL-23p19 may have ameliorate the inflammatory status of MG thymus by modulating the development of pathogenic Th17 cells without affecting the Treg cells population.

Thymectomy ameliorates thymic inflammation but not peripheral inflammation

Here I have shown that anti-IL-23p19 improves clinical symptoms in NSG-MG mouse model, while reducing inflammation in the thymus and to a lesser extend in the periphery. However, the reduced effects in periphery show that the thymus released pathogenic components that in the case of thymectomized patients probably remain in the periphery and must be controlled. Although, therapeutic thymectomy has been long used to improve clinical symptoms of MG, it does not induce remission, and some patients do not present a beneficial effect. In recent years the MGTX clinical trial showed that indeed thymectomy was effective to decrease MG symptoms as well as doses of corticosteroid in patients with AChR$^+$ antibodies [410]. However, this study included patients with a median disease duration at thymectomy of 1.08 years. Therefore, we cannot rule out that thymectomy could be less efficient in patients with longer duration of the disease before thymectomy. In a subsequent study to MGTX clinical trial, histological analysis of the recovered thymus was not able to establish a correlation between the clinical symptoms or the prospective reduction of medication intake after thymectomy with the degree of follicular hyperplasia, the hallmark of AChR$^+$ MG [482]. Hence, a closer analysis of the physiopathology of the thymus (TEC, thymocytes, DC and B-cells) might be carried out to determine the critical factor within MG thymus that could be linked to the patient's clinical status. Hence, numerous questions remain: May the thymic IL-23/Th17 pathway induces pathway related IgG production and IgG modifications that may correlate with disease clinical symptoms? What are the factors modified by the thymic IL-23/Th17 pathway (Th17 cells, induced B cells, cytokines, IgG) that are transferred to the periphery and maintained disease while thymectomy is already done?

Furthermore, there is a group of refractory MG patients that are not responsive or present limitations to the common therapies to treat AChR$^+$ MG (i.e. Corticosteroids, immunosuppressors and thymectomy). Refractory MG accounts for approximately 10% of the total cases of MG [458]. The consensual treatment for refractory MG patients is Rituximab, a monoclonal antibody that targets CD20$^+$ B-cells (anti-CD20). A recent retrospective study showed that, Rituximab ameliorate only 50% of the patients [483]. Even though this study displays limitation due to the MG cohort, a heterogeneous population that includes thymectomized and non- thymectomized patients and some patients affected with thymoma, it brings interested data. For instance, patients that underwent thymectomy still present clinical symptoms of MG and can respond to rituximab, illustrating that pathogenic cellular components of MG thymus released to the periphery may remained active long after thymectomy and be deleterious. Then, various reports have tried to link the peripheral inflammation with Th17 pathway. So far, studies are not conclusive. For instance, two studies showed that serum levels of IL-17 does not significantly differed between MG patients and healthy controls [271, 484]. In contrast, two more studies in Chinese and Spanish populations showed that MG patients had a significant increased concentration of IL-17 in serum and that the concentration of IL-17 was correlated with their clinical scores [421, 422]. More, one study showed that MG patients present a significant increase in the percentage of circulating Tfh cells with a Th17 cell phenotype (cTfh-Th17) that correlates with anti-AChR antibodies concentrations [485]. Here, I showed that MG patients present an increased level of IL-23 in serum. Therefore, after thymectomy, the Th17/IL-23 pathway could still play a role in the peripheral processes occurring in MG and supporting the hypothesis of treating patients with anti-IL-23.

In addition to IL-23, IL-6 is another cytokine involved in the development of Th17 cells. Tocilizumab is an anti-IL6R antibody that is used for the treatment of RA [466]. Recently a two cases were reported of refractive MG patients receiving tocilizumab [486]. These patients had been thymectomized and treatment with immunosuppressors and rituximab were inefficient. The report showed that in both cases Tocilizumab was well tolerated and induced an amelioration measured by the quantitative myasthenia gravis score (QMG) [486]. Although the physiological mechanisms by which tocilizumab improves MG patients is not described, this report set a precedent that new biological therapies that targets a cytokine related to Th17 cells could be efficient to improve MG.

Effects of IL-23p19 on MG muscle

The clinical analysis of EAMG mice that I carried out, showed that anti-IL-23p19 improved the clinical score two weeks after the initial dose and showed less inflammation highlighted by a reduction of IL-6 in the muscle. Interestingly at the third week of treatment, mice significantly reduced their clinical score to the levels close to CFA control mice. The clinical score improvement can be attributed to the significant decreased of TAChR antibodies IgG1 and IgG2b. These results are in line with previous results that showed that reducing the impact of the IL-23/Th17 cell pathway in EAMG mouse promote a decrease in antibody production [94, 479, 480].

As shown previously, in EAMG model, autoantibodies can modify the physiology of muscle and promote and inflammatory state as well as an activation of satellite cells [439, 440]. For instance, muscle from MG patients present an increased production of IL-6 induced by the presence of anti-AChR antibodies. Interestingly, in vitro experiments have shown that anti-AChR antibodies modified the Akt pathway (involved in muscle growth and glucose intake) in myotubes but not in myoblast [439], exhibiting that anti-AChR antibodies affected more differentiated muscle cells. In EAMG model, muscle regeneration has been shown to be decreased. Anti-AChR antibodies modify muscle satellite cells and muscle regeneration [440]. Indeed, after injury, EAMG muscle is capable to activate $Pax7^{+}$ satellite cells, however the differentiated myotubes present a decreases fusion index which translated into a more clinical symptoms [440].

I show that anti-IL-23p19 treated MG mice that display lower concentration of anti-TAChR, harbor in consequence a reduced muscular inflammation and limited activation of satellite cells. Of note, skeletal muscle regeneration requires satellite cells to downregulate Pax7 and MyoD and upregulate MyoG to differentiate in myotube and fuse to myofibers [433]. I observed that anti-IL-23p19 treated MG mice had in their tibialis anterior muscle a significant decrease expression of Pax7 and MyoD and an increased level of MyoG. Thus, it is possible that a limited inflammatory state of the muscle promotes muscle regeneration. Thus, our results showed that muscle alterations might be "corrected" in mice treated with anti-IL-23. However, the phenotypical and molecular analyses of the muscle myofiber remain to be analyzed.

CONCLUSIONS

In conclusion, this work showed that IL-23p19 is a critical cytokine in the inflammatory process of AChR$^+$ MG thymus and a potential therapeutic target to be consider in AChR$^+$ MG. New therapeutic approaches are emerging to treat AIDs, among them biological therapy with monoclonal antibodies. Here, I showed for the first time that anti-IL-23p19 monoclonal antibody can have a beneficial impact in the inflammation observed in human MG thymus by using the NSG-MG mouse model. I also showed in the classical MG mouse model that anti-IL-23p19 reduced the production of antibodies which translate into a less inflammatory status of the skeletal muscle. Therefore, with these approaches I showed that IL-23p19 can have a positive impact in both the thymus and the muscle to ameliorate MG symptoms. Therefore, my results must encourage to test other therapies than glucocorticoid and immunosuppressors to treat and to achieve higher number of AChR MG remission cases.

IV. PERPECTIVES

In this worked, I showed by using the two main pre-clinical models of MG, that the IL-23Th17 cell pathway plays a critical role in disease pathogenesis processes (thymus and muscle) and can be a new therapeutic target to ameliorate the clinical and global symptoms of MG by reducing thymic inflammation. However, some questions remain and new have emerged that remain to be investigated.

1- I have showed that IL-23p19 is a critical cytokine involved in MG. However, **in our NSG-MG model, we did not observe a decreased in the concentration of anti-AChR antibodies.** Therefore, it may be interesting to combine an anti IL23 therapy with a sparing to target autoantibodies such as eculizumab, an anti-C5 antibody that blocks the complement pathway and prevents the damage at the neuromuscular junction.

2- **Why some patients can present severe clinical symptoms while presenting low concentrations of autoantibodies?** It is possible that post-translational modifications of anti-AChR play a critical role in the pathogenicity of antibodies.
Since, we showed that Th17 cells are present around eGC in MG thymus, we may imagine that cytokines produced by these cells can influence antibody production by B cells and in consequence their pathogenicity. In this line, it would be interesting to explore the glycosylation status or other modifications of autoantibodies in MG, their potential relationship with the presence of Th17 cells and whether posttranslational modification of IgG are related to the clinical symptoms observed in MG.

3- Thymectomy is an effective but no curative procedure. The **beneficial effects of Rituximab in patients that underwent thymectomy indicates that cellular components of the thymus are released to the periphery.** Thus, it remains to be analyzed and to be determined the deep phenotypic and functional profile of the peripheral before and after thymectomy. This could provide important insights into the pathogenic components released by the thymus and therefore to assess and to adjust the therapeutic approaches and in order to achieve a higher beneficial effect.

4- **AChR$^+$ MG T cells display functional abnormalities** such as Treg harboring defective suppressive function. More Gradollato et al. have demonstrated that MG T cells display plasticity with Treg expressing and mimic Th17 cells features. Our results in the NSG-MG model showed a decreased number of Th17 cells but no effects on Treg cells number. Therefore, to go further, single cell analysis could provide information on whether anti-Il-23p19 in MG thymuses modifies or ameliorates molecular mechanisms involved in the development, the functional activities as well as in the plasticity of Treg cells.

5- **The therapies that target the IL-23/Th17 cell pathway display side effect** with patients can present a higher incidence of infections. Thus, in my thesis project, we did not observe or investigated this inherent consequence of the therapy. Therefore, it remains to be evaluated whether our therapy reduced Th17 cells to a level where infections could become critical. In this line, different doses of anti-IL-23 should be studied to achieve an equilibrium between the control of autoreactive Th17 cells and the preservation of the th17 cell protective role against infections.

6- The **role of IL-17 in MG muscle** is intriguing. Most reports of muscle IL-17 expression are associated to T cell infiltration. So far, MG muscle features and the crosstalk with the Th17/IL-23 pathway have not been deeper defined or characterized. Hence more studies have to be carried on identifying IL-17 function in MG muscle.

V. BIBLIOGRAPHY

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VI. ANNEXES

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ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Myasthenia Gravis and Related Disorders*
REVIEW

An imbalance between regulatory T cells and T helper 17 cells in acetylcholine receptor–positive myasthenia gravis patients

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A chronic autoimmune disease, myasthenia gravis (MG) is characterized in 85% of patients by antibodies directed against the acetylcholine receptor (AChR) located at the neuromuscular junction. The functional and effective balance between regulatory T cells (T_{reg} cells) and effector T cells (T_{eff} cells) is lost in the hyperplastic thymus of MG patients with antibodies specific for the AChR ($AChR^+$ MG patients). The objective of this review is to describe how T_{reg} cells and inflammatory T cells participate in this imbalance and contribute to induce a chronic inflammatory state in the MG thymus. We discuss the origins and characteristics of T_{reg} cells and their reported dysfunctions in $AChR^+$ MG patients. We also review the inflammatory condition observed in MG thymus, including overexpression of interleukin (IL)-1β, IL-6, and IL-23, cytokines that promote the differentiation of T helper 17 (T_H17) cells and the expression of IL-17. We summarize the preclinical models used to determine the implication of expression of cytokines, such as IL-6, IL-12 (IL-23 subunit), IL-17, and interferon γ to the development of experimental autoimmune MG. Finally, we suggest that biological agents, such as humanized monoclonal antibodies that target the IL-23/T_H17 pathway, should be investigated in the context of MG, as they have proven efficiency in other autoimmune diseases.

Keywords: autoimmune diseases; T regulatory cells; T_H17 cells; interleukin 17; interleukin 23; biological therapies

The immune system is highly ordered and relies on the balance between proinflammatory and anti-inflammatory signals and cellular responses. When the anti-inflammatory system does not display an adequate response, an exacerbated reaction occurs that can eventually lead to a pathological response, resulting in inflammation and autoimmunity.[1] The characterization (function and numbers) of the proinflammatory T cells (T helper 17 cells (T_H17 cells))[2–4] and the anti-inflammatory regulatory T cells (T_{reg} cells)[5] and their involvement in the promotion of various autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and psoriasis,[6,7] have been widely addressed. In these pathologies, an increased production of interleukin (IL)-17, a cytokine classically expressed by T_H17 cells, is observed,[8] as is, in many of them, defective T_{reg} cells.[9]

Myasthenia gravis (MG) is a chronic inflammatory autoimmune disease characterized by the presence of antibodies that target different protein components of the neuromuscular junction. Antibodies directed against the acetylcholine receptor (AChR) are present in 85% of MG patients, who also usually present muscle weakness and fatigability.[10] Interestingly, even if the symptoms of AChR-positive ($AChR^+$) MG patients are related to muscle fatigability, the thymus is the tissue in which the inflammatory process and the production of antibodies take place.[2] In young $AChR^+$ MG patients, the thymus displays follicular hyperplasia with ectopic germinal centers (eGCs) containing B cells,[11] development of high endothelial venules and lymphatic vessels,[12] and overexpression of chemokines such as CXCL13 and CCL21.[2]

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Various groups have demonstrated that hyperplastic MG thymuses have increased expression of proinflammatory cytokines such as IL-6, IL-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, as well as the anti-inflammatory cytokine transforming growth factor (TGF) β1.[13–16] In addition, in AChR$^+$ MG thymuses, MG patients do not have the normal equilibrium between T_{reg} cells (CD4$^+$ CD25$^+$) and T_H17 cells (CD4$^+$ CCR6$^+$ IL-23R$^+$ IL-17$^+$): T_{reg} cells are defective in their regulatory function[17] and can express markers of T_H17 cells, while T_{eff} cells (CD4$^+$ CD25$^-$) are resistant to suppression.[16]

Regulatory T cells

T_{reg} cells (CD4$^+$ CD25$^+$ FOXP3$^+$) are anti-inflammatory key players in the protection against autoimmunity.[18] Two major T_{reg} subsets, based on tissue origin, have been discriminated: thymic T_{reg} (tT_{reg} cells) or natural T_{reg} cells, and the peripheral T_{reg} (pT_{reg} cells) or induced T_{reg} cells.[19] Thymic T_{reg} cells constitutively express FOXP3 and CD25, the α-subunit of the IL-2 receptor.[20] In the thymus, medullary thymic epithelial cells participate in the generation of CD4$^+$ CD25$^+$ T_{reg} cells through the production of inducible T cell co-stimulator ligand (ICOSL). In CD4$^+$ T_{eff} cells, ICOSL induces the overexpression of IL-2, which promotes the generation of CD4$^+$ CD25$^+$ tT_{reg} cells.[21] In contrast to tT_{reg} cells, peripheral T_{reg} cells express FOXP3 and CD25 only after activation and require the presence of TGF-β.[22] The regulatory mechanisms of T_{reg} cells required to suppress effector cell proliferation are various, including cell–cell contact through the expression of the cytotoxic T lymphocytes–associated protein 4 (CTLA4), deprivation of IL-2, expression of anti-inflammatory cytokines (TGF-β and IL-10), or expression of granzyme A and activation of the perforin pathway.[7,23]

Within the population of FOXP3$^+$ T_{reg} cells, three subpopulations can be discriminated on the basis of their suppression capacity. CD45RA$^+$ FOXP3^{low} and CD45RA$^-$ FOXP3$^+$ T_{reg} cells display high suppressive capacity, while CD45RA$^-$ FOXP3^{low} T_{reg} cells are nonsuppressive.[24]

Development of autoimmune diseases can be considered in part to be caused by a deficiency in the number of T_{reg} cells and/or defective suppression mechanisms. In autoimmune diseases such as psoriasis, RA, and MS, T_{reg} cells are dysfunctional.[9,25,26] These defects cause a favorable environment for inflammatory cells to develop. In other diseases, such as inflammatory bowel disease, while T_{reg} cells may have a normal suppression capacity, T_{eff} cells are more likely to be resistant to suppression.[9] In autoimmune diseases, evaluation of T_{reg} cell populations must take into account cell number, detailed phenotype, and functional efficiency.

Regulatory T cells in myasthenia gravis

Balandina *et al.* showed that in the thymus of AChR$^+$ MG patients, the number of CD4$^+$ CD25$^+$ T_{reg} cells does not change compared with either age-matched healthy patients or newborn controls.[17] Another publication also showed no difference in the percentage of CD4$^+$ FOXP3$^+$ T_{reg} cells in the thymus or peripheral blood between healthy controls and MG patients with thymic hyperplasia.[27] However, in a recent analysis, Kohler *et al.* found a decreased percentage of CD45RA$^+$ FOXP3^{low} T_{reg} cells in MG patients, compared with healthy controls, and suggested that this may contribute to the disease course.[28] It is worth mentioning that owing to the T cell plasticity upon activation and/or cytokine environment, CD4$^+$ CD25$^-$T cells may switch to a CD4$^+$ CD25$^+$ phenotype.[29] Data provided by Huang *et al.* demonstrated that CD4$^+$ CD25$^-$ cells from MG patients could become CD4$^+$ CD25$^+$ induced T_{reg} cells that also express FOXP3 upon exposure to IFN-γ in a dose-dependent manner.[30] Therefore, analysis of the number of T_{reg} cells based only on their expression of CD25 may be a reason for the conflicting observations.

Although there is no consensus on there being a decreased percentage of T_{reg} cells in MG patients, most studies report that these cells have reduced suppressive activity. That is, the capacity of tT_{reg} cells from MG patients to suppress the proliferation of T_{eff} cells is highly impaired,[17] a feature also found in AChR$^+$ MG peripheral blood T_{reg} cells (defined as CD4$^+$ CD25^{high} CD127$^{low/-}$).[27] Of note, decreased expression of FOXP3 in MG thymus[17] and peripheral blood[27] can account for one of the causes of the impaired suppression capacity. FOXP3 expression is in part controlled by the phosphorylation of signal transducer and activator of transcription 5 (STAT5) induced by the IL-2 transduction pathway.[31] Reports have shown that MG patients present increased serum and thymic levels of IL-2,[32] while thymic and peripheral blood

lymphocytes have augmented the sensitivity to IL-2.[33] Low levels of FOXP3 in T_{reg} cells from MG patients may not, therefore, result from a deficiency of IL-2 but from decreased phosphorylation of STAT5 after IL-2 signaling.[34]

The implication of T_{reg} cells in MG thymic pathophysiological events has led to attempts to ameliorate disease course using T_{reg} cell therapy. For instance, in the animal model of MG (experimental autoimmune myasthenia gravis (EAMG)), Aricha *et al.* developed a cellular therapy by using *ex vivo*–expanded autologous $CD4^+$ $FOXP3^+$ T cells; they performed *ex vivo* expansion of T_{reg} cells from $CD4^+$ T cells cocultured with bone marrow–derived dendritic cells. Injection of expanded T_{reg} cells could reduce the clinical score of EAMG rats,[35] an improvement likely due to reestablished balance between T_{eff} and T_{reg} cells.

Recent studies of T_{reg} cells have provided evidence of another subpopulation of T cells known as T follicular regulatory cells (T_{FR} cells),[36] $CD4^+$ $CXCR5^+$ $FOXP3^+$ T cells derived from tT_{reg} cells. T_{FR} cells are involved in the control of T follicular helper cells (T_{FH} cells) and B cell responses and activation within germinal centers (GCs).[37] The mechanisms of regulation of T_{FR} cells are similar to those of T_{reg} cells, including expression of anti-inflammatory cytokines and CTLA4 and activation of the perforin pathway.[9,23,36] T_{FR} cells can also regulate class-switch recombination in B cells, achieved by inhibiting the activation-induced cytidine deaminase, a key enzyme in the class-switch recombination process.[37] Another hypothesis proposes that T_{FR} cells might control GC reactions by interacting with B cells and mechanically disrupting the contacts between T_{FH} cells and B cells.[36]

T_{FR} cells and T_{FH} cells share common characteristics, as they both express CXCR5, PD-1, ICOS, and Bcl-6. T_{FH} cells originate from naive $CD4^+$ T cells and do not express FOXP3.[36] T_{FH} cells are present in eGCs and stimulate B cell maturation, somatic hypermutation, and antibody production;[36] this is achieved through the expression of IL-21, IL-4, and IFN-γ and continuous cell–cell interaction that requires ICOS, CD40L, and CTLA4.[38,39] The interactions between T_{FH} and B cells stabilize GCs, and consequently the production of antibodies by B cells.[40]

T_{FH} cells and T_{FR} cells can also be found in the peripheral blood of various autoimmune diseases such as RA, MS, systemic lupus erythematosus, and MG. In the peripheral blood of MG patients, the number of $CD4^+$ $CXCR5^+$ $FOXP3^+$ T_{FR} cells decreases, while that of $CD4^+$ $CXCR5^+$ $FOXP3^-$ T_{FH} cells increases.[41] One report claimed that circulating levels of T_{FR}-like cells might be correlated with clinical progression of MG, and considered these cells to be a predictive marker of the course of MG.[41] Moreover, MG patients treated with glucocorticoids have a balance between $CD4^+$ $CXCR5^+$ $FOXP3^+$ T_{FR} and $CD4^+$ $CXCR5^+$ $FOXP3^-$ T_{FH} cells, as their percentages are similar to control patients.[41] Although the study provides insight into these two lymphocyte subpopulations in MG patients, there remains a lack of information about their participation events occurring in the thymus of MG patients.

Regulatory T cells within an inflammatory environment may also acquire altered T helper cell phenotypes.[42] For example, it was reported that purified $CD4^+$ $CD25^+$ tT_{reg} cells from $AChR^+$ MG patients express T_H17-related genes.[32]

T_H17 cells

T_H17 cells, extensively studied in autoimmune diseases and often inflammatory, are $CD4^+$ T cells characterized with the phenotype $ROR\gamma t^+$ $CCR6^+$ $IL\text{-}23R^+$ $IL\text{-}17^+$.[43] The differentiation of T_H17 cells develops in three overlapping steps directed by cytokines.[44] The differentiation of naive $CD4^+$ T cells into T_H17 cells after activation is through the effects of TGF-β1, IL-6, IL-21, IL-1β, and IL-23.[45] First, TGF-β1, IL-6, and IL-1β induce the phosphorylation of STAT3 and then subsequent activation of RORγt (the T_H17 cell signature transcription factor) in naive T cells. Second, $CD4^+$ $ROR\gamma t^+$ cells express IL-21 and IL-1β, which amplifies the T_H17 phenotype and induces expression of the IL-23 receptor (IL-23R). Finally, IL-23, a cytokine composed of two different subunits (IL-12p40 and IL-23p19), stabilizes the T_H17 phenotype and promotes the expression of IL-21, IL-22, and IL-17.[44,45] $CD4^+$ T cells that differentiate in the presence of IL-23 are considered pathogenic T_H17 cells (IL-23/T_H17 pathway) that overexpress IL-17[43] and are often involved in the pathogenic processes of autoimmune diseases.[46]

The IL-17 cytokine family comprises six members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F); IL-17A and IL-17F are highly homologous and the best-studied forms. The biological activity

of IL-17 is induced through its interaction with the IL-17 receptor (IL-17R), which is widely expressed in epithelial cells, fibroblasts, endothelial cells, and osteoblasts.[46,47] IL-17 induces the expression of chemokines, such as CXCL1, CXCL2, CCL2, CCL7, and CCL20, and proinflammatory cytokines, such as IL-6, TNF-α, IL-1β, and granulocyte colony-stimulating factor.[46] IL-17 contributes to the inflammatory process by attracting neutrophils and $CCR6^+$ cells, notably T_H17 cells.[48] IL-17 also contributes to the formation of eGCs[49] by helping B cell migration and inducing antibody class switching in GCs. Additionally, IL-17A synergizes with TNF-α, IL-1β, and IL-22 to expand the inflammatory process.[48]

T_H17 cells are normally found in mucosal barriers such as in the lungs and gut, where they participate in the clearance of pathogens to maintain tissue or organ homeostasis.[47] T_H17 cells found in nonbarrier organs are considered pathogenic and are usually linked to the development of autoimmune diseases. For example, pathogenic T_H17 cells and high levels of IL-17 can be found in psoriatic skin, RA synovium, and the cerebrospinal fluid in MS.[50,51] No report has yet clearly identified pathogenic T_H17 cells in the thymus of $AChR^+$ MG patients, although overexpression of the IL-17 family (IL-17A, IL-17F, IL-21, IL-22, and IL-26) has been shown in $AChR^+$ MG hyperplastic thymuses.[16] In addition, thymic T cells from $AChR^+$ MG patients overexpress IL-23 receptor (IL-23R), a marker of T_H17 cells,[32] implying a role for T_H17 cells in MG.

Various approaches to identifying or characterizing T_H17 cells in MG patients have been performed in peripheral blood and sera. A study by Cao *et al.* used a $CD4^+$ T cell library derived from MG patients and controls. They showed that AChR-reactive $CD4^+$ T cells from MG produced high levels of IFN-γ and IL-17, suggesting a mixed phenotype of T_H1/T_H17 cells.[52] T_H1 and T_H17 differentiation are linked to each other; they require IL-12 and IL-23, cytokines that share the common subunit IL-23p40, essential to activate the downstream signaling pathway. T_H17 cells are highly plastic and may become T_H1-like cells. In a milieu low in IL-23 or high in IL-12 and TNF-α,[53] T_H17 cells may limit their expansion and become IFN-γ–expressing cells.[54] Such T_H1-like cells, expressing IFN-γ and derived from T_H17 cells, have been described as more pathogenic than the originated T_H17 cells.[53] Induction of inflammatory autoimmune disease may occur upon the secretion of pathogenic cytokines, such as IL-17, GM-CSF, and IFN-γ, potentially resulting in a balance between T_H1-like/T_H17 autoreactive T cell activation, as suggested by Cao *et al.*[52]

A second group reported no significant differences in the levels of proinflammatory cytokines in T_H17 and T_H1 cells produced by $AChR^+$ MG peripheral blood mononuclear cells stimulated with anti-CD3 antibodies, compared with controls.[55] They also reported similar levels of plasma IL-17 between $AChR^+$ MG patients and healthy controls.[55] Another study carried out on a Japanese population showed similar results, with no differences in the serum levels of IL-17 and IFN-γ between MG patients and healthy controls.[56] Two additional studies evaluated the levels of IL-17 in serum[57] and plasma[58] and found that MG patients have a significantly increased concentration of IL-17. In addition, in the thymus of $AChR^+$ patients, overexpression of IL-17 has been reported,[16,45] suggesting a potential thymic activation of T_H17 cells. This hypothesis was reinforced by the findings of Gradolatto *et al.*, which showed that, in thymocytes of MG patients, there is overexpression of IL-17–related genes and IL-23R.[32]

In summary, $AChR^+$ MG hyperplastic thymuses exhibit many features of inflammation related to the IL-23/T_H17 pathway that are classically observed in other autoimmune diseases, emphasizing the importance of understanding the involvement of the imbalance between T_{reg} cells and T_H17 cells in $AChR^+$ MG thymuses.

Cytokines related to T_H17 cells and experimental autoimmune myasthenia gravis

To decipher the impact of T_H17/T_{reg} cells on MG disease course, experiments using animal models have been performed. The classical experimental animal model of MG (EAMG) is based on the immunization of mice with AChR, isolated from the electric organs of the *Torpedo* fish, in complete Freund's adjuvant. EAMG mimics some MG symptoms: mice develop weakness, anti-AChR antibodies, and evidence of IgG deposits at the neuromuscular junction.[59] Even though the thymus is

not implicated in this model, some immunological changes can be observed in the spleen.[60]

EAMG studies with knockout (KO) mice have shown that cytokines related to the T_H17 pathway play critical roles in the development of EAMG. For instance, IL-6 and IL-17 gene KO mice present a lower EAMG incidence and less severe clinical symptoms than their respective wild-type (WT) mice.[61–63] Additionally, in both models, there is a reduction of the level of the anti-AChR antibodies, especially the IgG_2b isotype, and a decreased GC area in the spleen of EAMG KO mice compared with controls.[61,62] Moreover, *in vitro* analyses showed decreased production of IFN-γ in AChR-stimulated lymphocytes from *Il6*$^{-/-}$ mice but not from IL-17–deficient (*Il17a*$^{-/-}$) mice,[61–63] suggesting that IL-6 may have a wider impact on the inflammatory process than IL-17.

The implication of IL-12 has also been studied in the context of EAMG. Karachunski *et al.* compared the development of EAMG in two different deficient mouse models, IL-12–deficient (*Il12a*$^{-/-}$) and IFN- γ–deficient (*Ifng*$^{-/-}$) mice. The absence of IL-12 protects mice from the development of EAMG, while IFN-γ has no significant effect.[64] *Il12a*$^{-/-}$ mice present with decreased levels of IFN-γ and low levels of anti-AChR IgG_2b antibodies, while *Ifng*$^{-/—}$ mice show the same amount of anti-AChR IgG_2b as WT controls.[64] The effect of the depletion of either IL-12 or IFN-γ on T_H17 cells and IL-17 has not been reported. In contrast, combined depletion of both IFN-γ and IL-12p40 increases the susceptibility to EAMG, probably because of a reduced function of T_{reg} cells.[65]

EAMG is the classic model by which we study MG. Although experiments with KO mice have provided important clues to how different cytokines related to T_H17 cells can participate in the development of EAMG, they cannot address the role of T_H17 cells in the thymus, since it is not involved in these mouse models. In fact, the immunization process induces the muscle weaknesses without affecting the thymus.[60] To overcome this limit, Sudres *et al.* developed a humanized preclinical model that recapitulates the features of human MG. In this model, subcutaneous transplantation of thymus fragments from MG patients in NOD/*scid* gamma mice induces MG symptoms that include fatigability and the presence of anti-AChR antibodies.[66] Additionally, in this model, the thymic fragments from MG patients conserve their inflammatory status, with overexpression of IL-6, IL-17, TNF-α, and IFN-γ.[66] This humanized MG mouse model may be relevant for determining the importance of different T cell subsets, especially T_H17 cells, in the disease course and for evaluating therapies targeting molecules of the IL-17/IL-23 pathway.

Therapeutic approaches in AChR$^+$ MG patients

To date, therapies for AChR$^+$ MG are largely focused on symptoms. The first-line therapy is based on inhibitors of acetylcholinesterase that induce the accumulation of acetylcholine at the neuromuscular junction and therefore reduce fatigability.[67] The second-line and most common treatments are nonspecific immunotherapies that include corticosteroids and/or immunosuppressants.[68] Thymectomy is a therapeutic option that provides significant amelioration for AChR$^+$ MG patients and allows corticosteroid adjustments.[69] Recently, rituximab, a monoclonal antibody that targets CD20$^+$ B cells, was tested in AChR$^+$ MG and showed effectiveness primarily in patients with anti–muscle-specific kinase antibodies.[68] A recent study has shown that AChR$^+$ MG patients resistant to immunosuppressive therapy can improve when treated with rituximab.[70] Even with therapies for treatment of MG that improve quality of life of patients and reduce mortality, more effective therapies with fewer side effects that specifically target the physiopathological changes present in MG patients are needed.

Understanding autoimmune diseases has highlighted the importance of the IL-23/T_H17 pathway in these pathologies. Patients refractory to conventional treatments have allowed the investigation of molecules that target the IL23/T_H17 pathway. Monoclonal antibodies that target IL-17, IL-17R, IL-12p40, and IL-23p19 are now being tested, or have been recently approved, to treat autoimmune diseases such as psoriasis, RA, MS, type 1 diabetes, and Crohn's disease.[46,71,72] Secukinumab and ixekinumab, monoclonal antibodies that specifically target IL-17A, have been approved for the treatment of psoriasis and are in phase III clinical trials for psoriatic arthritis.[72] Multiple studies have shown that secukizumab and ixekizumab inhibit the expression of a wide variety of genes associated with T_H17-mediated inflammatory responses by limiting the activation of keratinocytes and immune cell

Table 1. Biological agents targeting the IL-23/T_H17 pathway

Biological agent	Target	Antibody type	Disease (clinical trial)
Secukinumab	IL-17A	Fully human IgG1	Psoriasis (approved) Psoriasis arthritis (approved) RA (phase III)
Brodalumab	IL-17RA	Fully human IgG2	Psoriasis (approved) Psoriasis arthritis (approved)
Ustekinumab	IL-12p40	Fully human IgG1	Psoriasis (approved) Psoriasis arthritis (approved)
Guselkumab	IL-23p19	Fully human IgG1λ	Psoriasis (approved) Psoriasis arthritis (phase II)

Note: Several monoclonal antibodies are now being tested and used in various autoimmune diseases. In myasthenia gravis, studies have shown the potential involvement of the IL-23/T_H17 pathway. Therefore, monoclonal antibodies that target cytokines or receptors linked to this pathway are possible options worth testing in MG.[72,74]

recruitment.[73] Both antibodies are effective long-term treatments for plaque psoriasis, although two phase III clinical trials reported that they may have serious adverse effects, such as stroke and myocardial infarction.[73]

Inhibiting the IL-17 receptor is another way to target the IL-17 pathway. Brodalumab, a monoclonal antibody that targets IL-17RA, has been approved in several countries for the treatment of psoriasis and psoriatic arthritis. However, clinical trials to treat RA and inflammatory bowel disease with brodalumab were discontinued owing to no evident clinical efficacy or disease exacerbation.[63,74] Of note, IL-17RA forms a dimeric complex that allows signal transduction by various cytokines, including IL-17A, IL-17F, IL-17A/F, IL-17C, and IL-25. Blocking all IL-17 isoform signaling, therefore, may be counterproductive in tissue-dependent disease where T_H17 cells play a protective role against opportunistic infections. The differentiation of pathogenic T_H17 cells is dependent on IL-23. Ustekinumab, a fully human monoclonal antibody that targets the p40 subunit of IL-12 and IL-23, is now available for patients with psoriasis, psoriatic arthritis, and Crohn's disease.[72,74] By contrast to the previous monoclonal antibodies, ustekinumab has shown efficiency for Crohn's disease patients.[75,76] Finally, a fully human-specific antibody that targets IL-23p19 has recently obtained U. S. Food and Drug Administration approval to be used as a therapy for patients with moderate-to-severe plaque psoriasis.

Table 1 summarizes the biological agents that target the cytokines overexpressed in MG and their potential use as treatments. At present, there are numerous listed clinical trials related to MG, but none aim to test monoclonal antibodies that target the IL-23/T_H17 pathway (clinicaltrials.gov). This is probably due to the lack of explicit and convincing data showing the implication of this pathway in the targeted tissue (muscle) or the effector tissue (thymus) of $AChR^+$ MG patients, a contrast to other autoimmune diseases that display tissue cell infiltrations and increased IL-17 levels.

Summary comments

The highly inflammatory conditions in the $AChR^+$ MG thymus include dysfunctional T_{reg} cells and T_{eff} cells resistant to suppression, which together are associated with altered immune regulation processes. In addition, increased levels of IL-17, IL-23p40, and T_H17–related gene expression may contribute to, initiate, or maintain the inflamed thymic environment. The contribution of IL-17–expressing cells within the thymus has yet to be determined, and doing so may help to clarify the involvement of IL-17–expressing cells and their relationship with the development or perpetuation of the inflammatory state in the $AChR^+$ MG thymus. Consequently, adequate or specific biological therapies, such as monoclonal antibodies that target the IL-23/T_H17 pathway—already described to be efficient in other autoimmune diseases—could be considered for treating MG.

Competing interests

The authors declare no competing interests.

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REVIEW

Thymus involvement in early-onset myasthenia gravis

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

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

Introduction

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

In contrast, no thymic abnormalities are observed in MG with muscle-specific kinase antibodies. In MG with LRP4 antibodies, thymic hyperplasia of lymphoproliferative origin has also been observed in a few patients but not further investigated so far.[2] Here, we mainly focus on thymic changes occurring in early-onset AChR$^+$ MG patients occurring usually before 45–50 year of age. Most of these MG patients present high level of anti-AChR antibodies and thymic follicular hyperplasia. Sex hormones may play a role in this form of the disease, as more than 80% of patients with follicular hyperplasia are women.

The normal thymus

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

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

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

Thymic abnormalities in early-onset MG

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

Abnormal T cell functionality

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

Peripheral cell infiltrations leading to germinal center development

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

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

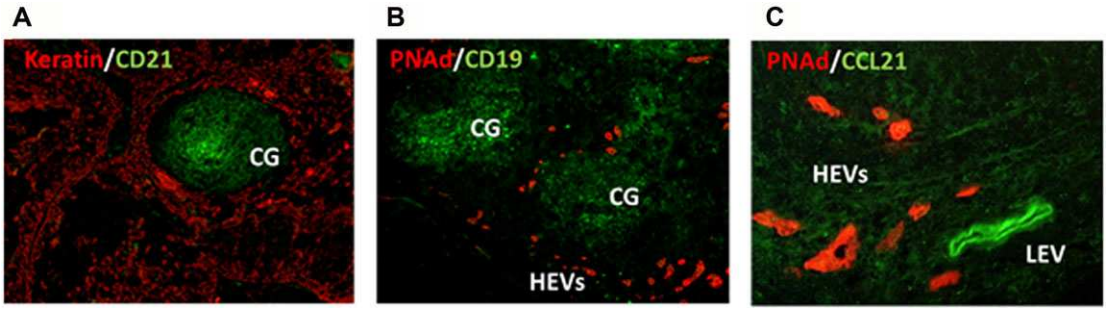

Figure 1. Thymic changes in MG patients. Thymic sections from MG patients were labeled (A) in red with an anti-keratin antibody and in green with an anti-CD21 antibody for B cells and follicular dendritic cells to localize GCs; (B) in red for HEVs with an anti-peripheral node addressin (PNAd) antibody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

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

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

Neoangiogenic processes

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

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

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

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

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

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

Chemokine overexpression

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

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

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

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

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

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

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

Pathogen infection signature associated with Toll-like receptor expression

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

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

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

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

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

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

Central role of interferon-β

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

IFN-I proteins, specifically IFN-β, induce the specific expression of α-AChR by TECs and not that of other TSAs. It also increases TEC death and the uptake by dendritic cells of TEC proteins, potentially the α-AChR that is overexpressed in IFN-β–treated TECs. In parallel, IFN-β triggers the expression of CXCL13 and CCL21 by TECs and lymphatic endothelial cells, respectively, and consequently could favor peripheral cell recruitment in the thymus. IFN-β also induces the expression of B cell activating factor (BAFF), which favors B cell survival and is overexpressed by TECs in MG thymus.[64] Similar changes are observed *in vivo*, as the injections of poly(I:C) to C57BL/6 mice trigger a thymic overexpression of IFN-β and IFN-α2 associated with increased expressions of CXCL13, CCL21, and BAFF and favor the recruitment of B cells. These changes are not observed in the thymus of IFN-I receptor gene knockout mice injected with poly(I:C).[64]

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

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

Dysregulated expression of miRNAs

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

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

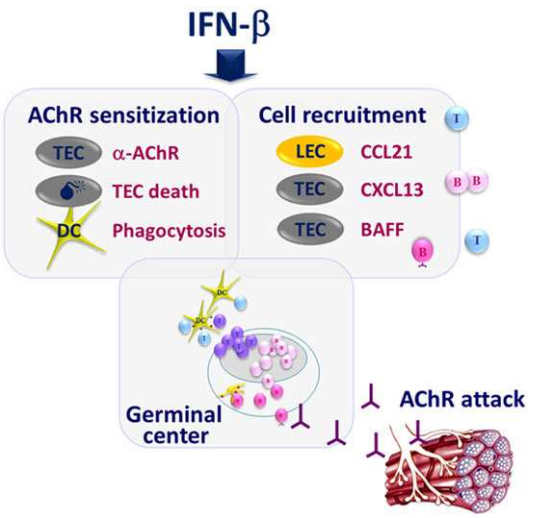

Figure 2. Central role of IFN-β in thymic changes associated with MG. IFN-β induces the expression in thymic epithelial cells (TECs) of α-AChR, the main autoantigen in MG. This effect is very specific to α-AChR, as IFN-β does not induce the expression of other AChR subunits or other tissue-specific antigens.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

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

Conclusions

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

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Competing interests

The authors have declared no competing interests.

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Balance between Estrogens and Proinflammatory Cytokines Regulates Chemokine Production Involved in Thymic Germinal Center Formation

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The early-onset form of Myasthenia Gravis (MG) is prevalent in women and associates with ectopic germinal centers (GCs) development and inflammation in the thymus. we aimed to investigate the contribution of estrogens in the molecular processes involved in thymic GCs formation. We examined expression of genes involved in anti-acetylcholine receptor (AChR) response in MG, MHC class II and α-AChR subunit as well as chemokines involved in GC development (CXCL13, CCL21and CXCL12). In resting conditions, estrogens have strong regulatory effects on thymic epithelial cells (TECs), inducing a decreased protein expression of the above molecules. In knockout mouse models for estrogen receptor or aromatase, we observed that perturbation in estrogen transduction pathway altered MHC Class II, α-AChR, and CXCL13 expression. However, in inflammatory conditions, estrogen effects were partially overwhelmed by pro-inflammatory cytokines. Interestingly, estrogens were able to control production of type I interferon and therefore play dual roles during inflammatory events. In conclusion, we showed that estrogens inhibited expression of α-AChR and HLA-DR in TECs, suggesting that estrogens may alter the tolerization process and favor environment for an autoimmune response. By contrast, under inflammatory conditions, estrogen effects depend upon strength of the partner molecules with which it is confronted to.

Myasthenia gravis (MG) is a heterogeneous neurological autoimmune disease caused by antibodies directed against proteins of the neuromuscular junction. In 85% of patients, antibodies are directed against the acetylcholine receptor (AChR)[1,2] and associated with thymic abnormalities including follicular hyperplasia and thymoma. Thymic follicular hyperplasia form affects mainly female patients (ratio 4:1) during the fecund period of their life[3]. Of note, thymus removal (thymectomy) completed in an early stage of the disease, is generally an efficient therapy inducing a gradual decrease in anti-AChR antibody titer in the serum and improving symptoms[4,5].

We and other groups have demonstrated that the biological hallmark of MG thymic hyperplasia is the presence of ectopic germinal centers (GCs)[6], which provide specific activated and differentiated B cells producing anti-AChR antibodies[7]. These features go along with dysregulated expression of CXCL13, CCL21, and SDF-1/CXCL12[8–10], chemokines that play a central role in lymphocyte trafficking to lymphoid and non-lymphoid tissues in both physiological[11] and pathophysiological[12] conditions.

In autoimmune diseases, the migration and the accumulation of lymphocytes in the target organs are important steps of the pathogenesis. Indeed, high levels of chemokines or cytokines are observed in blood or tissues of patients and correlate with the severity of the disease[8,9,13,14]. Moreover, the ectopic expression of CXCL13 and CCL21 in transgenic mice has been reported to be sufficient to induce lymphoid neogenesis, which leads to the

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formation of lymph node-like structures[15]. Also, we have recently demonstrated that inflammation is required to reinforce CXCL13 function in recruitment and induction of B cells in tertiary lymphoid organ development[16].

Interestingly enough, hyperplasic MG thymuses display signs of inflammation with an increased expression of interferon (IFN) type I and type II regulated genes including HLA-DR genes[17].

Autoimmune MG as compared to most autoimmune diseases (AIDs) are more prevalent in women than men[18]. The reason for this gender incidence was not understood for a long period. Recently, various investigators brought data to light up this phenomenon. Hence, we have demonstrated that the female main sexual hormone, estrogens contribute to the gender bias female susceptibility to AIDs by partially silencing the autoimmune regulator (AIRE)[19]. In addition, estrogens display various known roles in humoral and cellular responses to infection and vaccination in men and women[20]. As well, another group has demonstrated that dihydrotestosterone (DHT), the main testosterone metabolite may have an opposite effect by stimulating in the thymus tolerance mechanisms, again highlighting the role of sexual hormones in AID susceptibility phase. While sex hormones contribute to disease etiology, they also modulate the activity of the immune system and consequently the evolution of AIDs. Therefore, during pregnancy or menstruations, steroid hormones favor a polarization of the immune response towards a Th2 response[21]. For instance in autoimmune MG patients, a worsening of the clinical symptoms during pregnancy or menstruations have been reported, a phenomenon that disappears after thymectomy[22]. Further, we have demonstrated that estrogen receptor subunit α (ER-α) is upregulated in thymocytes of MG patients[23], suggesting a possible role of sex hormones in thymic pathogenesis and pathology incidence.

Women affected with autoimmune MG are more prone to develop hyperplasic thymuses[3]. Also, and as indicated above, less efficient thymic tolerance process could contribute to the higher female susceptibility to autoimmune MG. Here, we attempt to understand why females display more frequently a hyperplastic thymic in MG, and the involvement of female sexual hormone, estrogens, in these physiopathological processes. To this end, we analyzed the influence of, estrogens on the expression of the chemokines CXCL13, CCL21 and CXCL12 that are highly attractive for B and T cells and are involved in GC formation[24,25], as well as molecules that participate in the mechanism of central tolerance including HLA class II antigens and AChR subunits.

Our data clearly indicate that estrogens induced a low steady state expression level of most molecules analyzed. However, in an inflammatory environment, comparable to events found in MG thymus, estrogens sustain the activation of interferon-signaling pathways enhancing the process of GC formation.

Results

Estrogen effects on HLA-DR and α-AChR expressions in thymus. To understand the molecular differences underlying the female prevalence in MG, we first investigated in normal thymuses from males and females the expression of α-AChR and HLA-DR molecules essential for central tolerance mechanisms, and for the autoantigen presentation in MG. We, then evaluated the effects of estrogens on the expression of these molecules at the mRNA and protein levels.

Analysis of whole normal human thymuses by real-time qPCR showed a gender differential effect with women expressing significantly less α-AChR but also slightly less HLA-DR compared to men (Fig. 1a,b). The effect of estrogens on the expression of these two molecules was analyzed in thymic epithelial cells that express AChR[26] and MHC class II[27], and are involved in thymic tolerance processes. Our data demonstrated that estrogens decreased the expression of α-AChR and HLA-DR proteins in cultured thymic epithelial cells (TECs) (Fig. 1c,d). This estradiol effect on the expression of α-AChR is significant at physiological doses (10^{-8} to 10^{-9}) as observed in the dose effect curve (Supplemental Fig. S1). Altogether, these data suggest that estradiol may participate in the tolerization process for α-AChR subunit by regulating its expression and that of HLA-DR molecules.

To confirm the influence of estrogens on the expression of these molecules, we analyzed their mRNA levels in thymuses of mice deficient in estrogen receptor-α (ER-α) (principal isoform found in TECs[23]), estrogen receptor-β (ER-β) or aromatase (ArKO) (the enzyme involved in estrogen synthesis). As shown in Fig. 1e, the level of α-AChR was low in ArKO mice and significantly higher in ER-α KO mice compared to WT and ER-β mice, suggesting that estrogen effects were mediated through its nuclear receptor ER-α for α-AChR subunit expression in TECs. By contrast, MHC class II expression was not altered in ER-α, ER-β KO mice, but increased in ArKO mice (Fig. 1f), suggesting that estrogens may induce an ER-independent transduction pathway to control MHC II expression.

Altogether these data corroborate what we have already published about women after puberty, that display a decreased capacity in TECs to express tissue-specific antigens regulated by autoimmune regulator among them α-AChR[19].

Estrogen effects on chemokine expression in the thymus. We then wondered whether women exhibit differences in signaling molecules underlying the physiopathologic events occurring in the thymus of MG patients. We conducted a pan-genomic expression analysis using the "Human 1 cDNA arrays from Agilent" to compare thymic tissues from young men and women as detailed in Dragin *et al.*[19]. Analysis of the expression of all chemokines included in the arrays showed a significant difference between men and women (paired analysis, $p < 0.01$) (Fig. 2a) while no significant difference was observed for the interleukin family (Fig. 2b) (Supplemental Table S1a and b). Among the 17 chemokines included in the array, 14 were more expressed in males than females (Supplemental Table S1a). Moreover, a similar analysis on clusters of differentiation markers (Supplemental Fig. S2a) and keratins (Supplemental Fig. S2b) that illustrates the distribution of T-cell populations and epithelial cells, respectively, was performed. No differential expression between men and women for these gene families was observed, suggesting that the difference in chemokine thymic expression between men and women was not due to differences in thymic cell populations. Besides, to validate the gender differential expression observed in the microarray, we performed by real-time qPCR on, CXCL13, CCL21 and CXCL12, chemokines involved in MG pathological process. We observed that CXCL13 and CCL21 expressions were significantly decreased in women

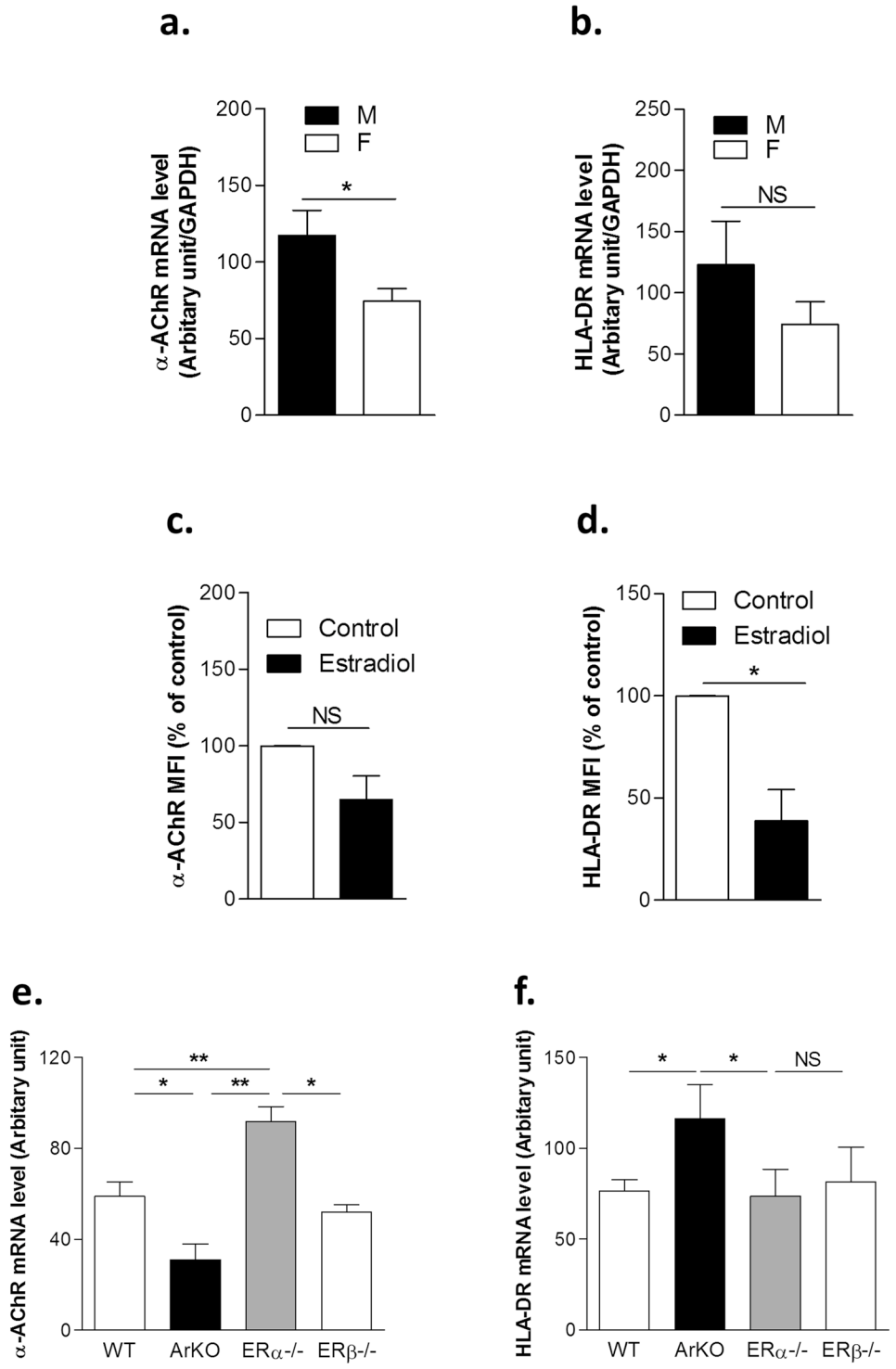

Figure 1. Modulation of α-AChR and HLA-DR expression in human and mouse thymuses and by β-estradiol in human primary cultured TECs. mRNA expression levels of α-AChR (**a**) and HLA-DR (**b**) in normal human male and female thymuses. Effect of 17-β estradiol (10^{-8}M) on α-AChR (**c**) and HLA-DR (**d**) protein expression in human primary TECs. mRNA expression levels of α-AChR (**e**) and MHC II (**f**) in thymuses of C57Bl6J, Aromatase knock-out (ArKO), estrogen receptor α KO (ERα−/−) and estrogen receptor β KO (ERβ−/−) female mice. ($n > 4$ for human and $n > 3$ for mouse thymuses). P values were obtained using Mann-Whitney test for (**a**,**b**); P values were obtained using the Wilcoxon test for (**c**,**d**); P values were obtained using the Student t test for (**e**,**f**).

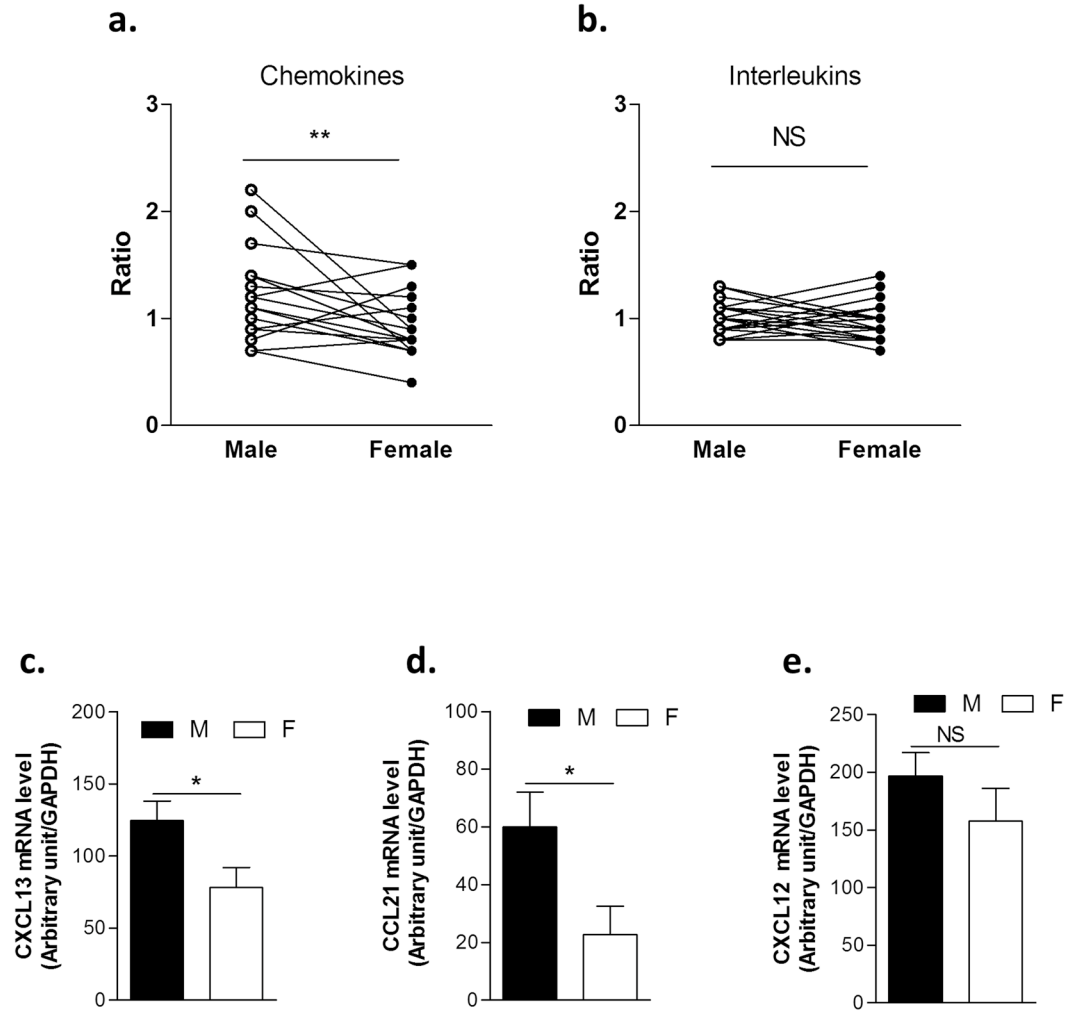

Figure 2. Analysis by microarray and RT-PCR of cytokine gene expression in male and female thymuses. Expression ratios of chemokine (**a**) and interleukin (**b**) genes spotted on the arrays for man and woman adults, compared to a thymic reference composed of thymuses from female babies. Each dot corresponds to the ratio of the median of five and four replicate arrays respectively for women, and men for a given gene. mRNA expressions of CXCL13 (**c**), CCL21 (**d**) and CXCL12 (**e**). $n > 4$ individual thymuses, aged from 17 to 44 years. P values were obtained using the Wilcoxon test for (**a**,**b**), and the Mann-Whitney test for (**c** to **e**).

as observed with the microarray results, while CXCL12 did not display gender bias expression in whole human thymuses of adult women compared to men (Fig. 2c–e).

Since our data showed a decreased expression of chemokines in female thymus, we then asked whether estrogens could be responsible for these reduced chemokine expressions.

As shown in Fig. 3, in human primary TECs, a physiological dose of estradiol (E2) induced a significant decrease in protein expression of CXCL13 while no effect was found for CCL21 (Fig. 3a,b respectively), suggesting that lower level of CXCL13 in females compared to males could be due to the effects of estrogens (Fig. 3a). Of note, estradiol effect on the expression of chemokines is significant at physiological dose (10^{-8} to 10^{-9} M) as observed in the dose effect curves (Supplemental Fig. S3). Indeed, we demonstrated that normal physiological doses inhibited the expression of α-AChR, CXCL13, CCL21 while they upregulated CXCL12 expression in primary human TECs. Surprisingly, although no gender differential expression was found for CXCL12, estrogens up-regulated in TECs, its protein expression (Fig. 3c). Of note, these data corroborate previous findings displaying, in other tissues, upregulation of CXCL12 by physiological concentrations of estrogen through ER-α[28,29].

In addition, the analyses of chemokine mRNA levels in the thymus of ER-α KO, ER-β KO or ArKO mice revealed that CXCL13 mRNA expression (Fig. 3d) was increased in ArKO mice but unchanged in ER-β KO mice. These data strongly suggest that CXCL13 transcript level was down-regulated by estrogens, independently to its ER-α nuclear receptor. By contrast, CCL21 expression was not altered in the mutated mice, suggesting that CCL21 expression is probably related neither to estrogens nor its nuclear receptors (Fig. 3e).

Effect of inflammatory signals with estrogens on HLA-DR, α-AChR and chemokine expression. We have previously demonstrated a chronic inflammation in MG hyperplastic thymuses characterized

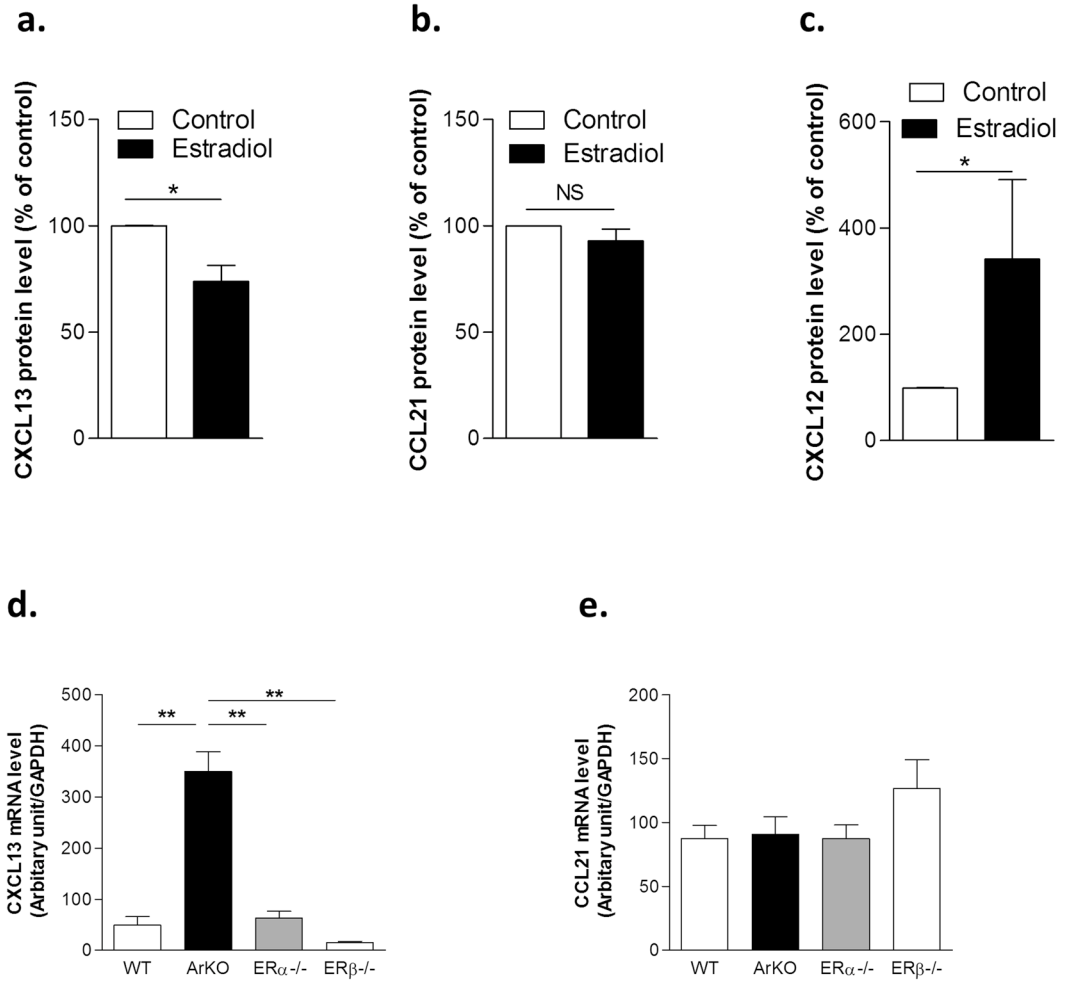

Figure 3. Modulation of chemokine expression in mouse thymuses and by β-estradiol in human primary cultured TECs. Human primary TECs were stimulated for 24 hours in the presence of 17-β estradiol (10^{-8}M). Effects of estradiol on CXCL13 (**a**), CCL21 (**b**) and CXCL12 (**c**) protein expression by cultured primary human TECs. mRNA expression levels of CXCL13 (**d**) and CCL21 (**e**) in thymuses of C57Bl6J, aromatase knock-out (ArKO), estrogen receptor α KO (ERα−/−) and estrogen receptor β KO (ERβ−/−) female mice. Results are the mean values ± SEM. n = 5 different TEC supernatants for protein analysis and n > 3 different mouse thymuses per strain. P values were obtained using the Wilcoxon test for (**a** to **c**); P values were obtained using the Student t test for (**d**,**e**).

by an overexpression of cytokines, in particular IFN γ and TNF α[30]. Since young female MG patients are prone to develop hyperplastic thymuses, we investigated here the interplay between estrogens and a pro-inflammatory environment. In order to mimic the inflammatory MG thymic condition that occurred in female.

In primary cultured human TECs, the inflammatory mix that mimics MG thymic environment[31] drove a significant expected and prominent upregulation of HLA-DR expression, an effect unchanged in the presence of estradiol (Fig. 4a). However, the cytokine mix combined with estradiol induced a reduced α-AChR expression (Fig. 4b).

The analysis of the chemokines at the mRNA level revealed that the cytokine mix caused an increased expression of CXCL13, CCL21, and CXCL12, and estrogens limited this increase (Fig. 4c–e). However, the changes at the protein level were less striking, except for CXCL13 that was significantly reduced in the presence of inflammatory cytokines (Supplemental Fig. S5). The apparent contradiction between the increased mRNA and decreased protein level could be due to a higher degradation or a reduced secretion of the proteins. Of note, the effect of single cytokine exposure was analyzed (Supplemental Fig. S4. We observed that IFNγ effect was the highest for CXCL13 and TNF-α and IL-1β were the highest for CXCL12. As previously observed, combined different cytokines lead to a stronger effect while cytokines are used in single. A synergistic or exacerbation is then observed on the cytokine expression.

We next wondered whether this modulation might impact or modify the chemoattractant capacity of human TECs. We found that supernatants from TECs treated with estrogens exhibited a significant decreased chemotactic activity on peripheral blood leukocytes (PBLs) (Fig. 5a), an effect likely due to diminished migration of T (Fig. 5b) and not of B lymphocytes (Fig. 5c). Altogether, these data demonstrated that estrogens could decrease

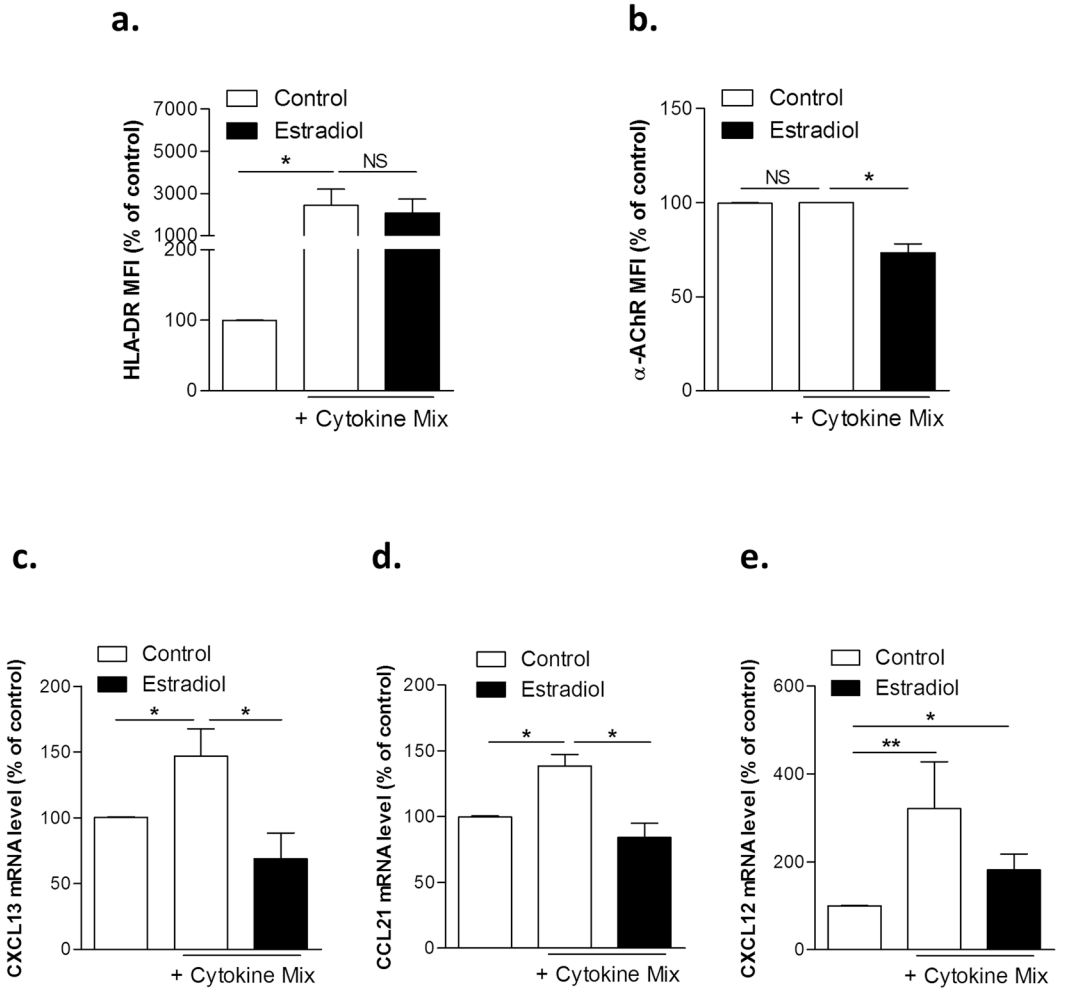

Figure 4. Modulation of HLA-DR, α-AChR, CXCL13, CCL21 and CXCL12 expression in human primary TECs by pro-inflammatory cytokine mix with β-estradiol. Effects of a cytokine mix with 17-β estradiol (10^{-8}M) on HLA-DR (**a**), α-AChR (**b**), CXCL13 (**c**), CCL21 (**d**) and CXCL12 (**e**) expressions in human primary TECs. HLA-DR and α-AChR protein levels were analyzed by flow cytometry. Primary cultured TECs were obtained from at least five different donors. P values were obtained using the Wilcoxon test.

chemokine expression by TECs and then as a consequence reducing TEC ability to recruit T cells. In the presence of the inflammatory mix, the inhibitory effect of estradiol on chemotactism was less pronounced on total PBLs but still significant on T lymphocytes (Fig. 5a–c).

Together, by controlling the expression of some MG-related molecules, even in inflammatory conditions, estrogens can limit the inflammation burst. Interestingly, the effect of estrogens was detected only when the inflammatory mix induced small expression changes.

Estrogens upregulate the expression of Type I interferon and related molecules in human primary thymic epithelial cells. The results above did not provide an explanation for the higher frequency of follicular hyperplasia in females that is associated with higher expression of CXCL13 and CCL21. Among molecules able to upregulate the expression of these chemokines, we previously reported that Type I interferon is a potent regulator of CXCL13 and CCL21 in primary cultured epithelial cells and lymphatic endothelial cells, respectively[32]. Others authors have demonstrated that IFN-I production or expression is regulated by estradiol[33] in natural killer cells[34], lymphocytes[35], and B cells[36]. Moreover, estradiol regulation of IFN-I signaling participates to the gender bias disease development as already demonstrated by Choubey et coll[37]. We thus asked whether estrogens could affect the expression of type I interferon in human primary TECs.

We observed that estrogens stimulated expression of IFN-α and -β, (Fig. 6a,b respectively). Moreover, interferon-related genes such as OAS2 and MXA displayed also, but to a lesser extent, an estrogen modulation effect (Fig. 6c,d). These observations corroborate findings observed in other cell types showing that estrogens can stimulate the type I interferon pathway[38]. These data demonstrate the complexity of estrogen effect and its diverse roles depending on the environment.

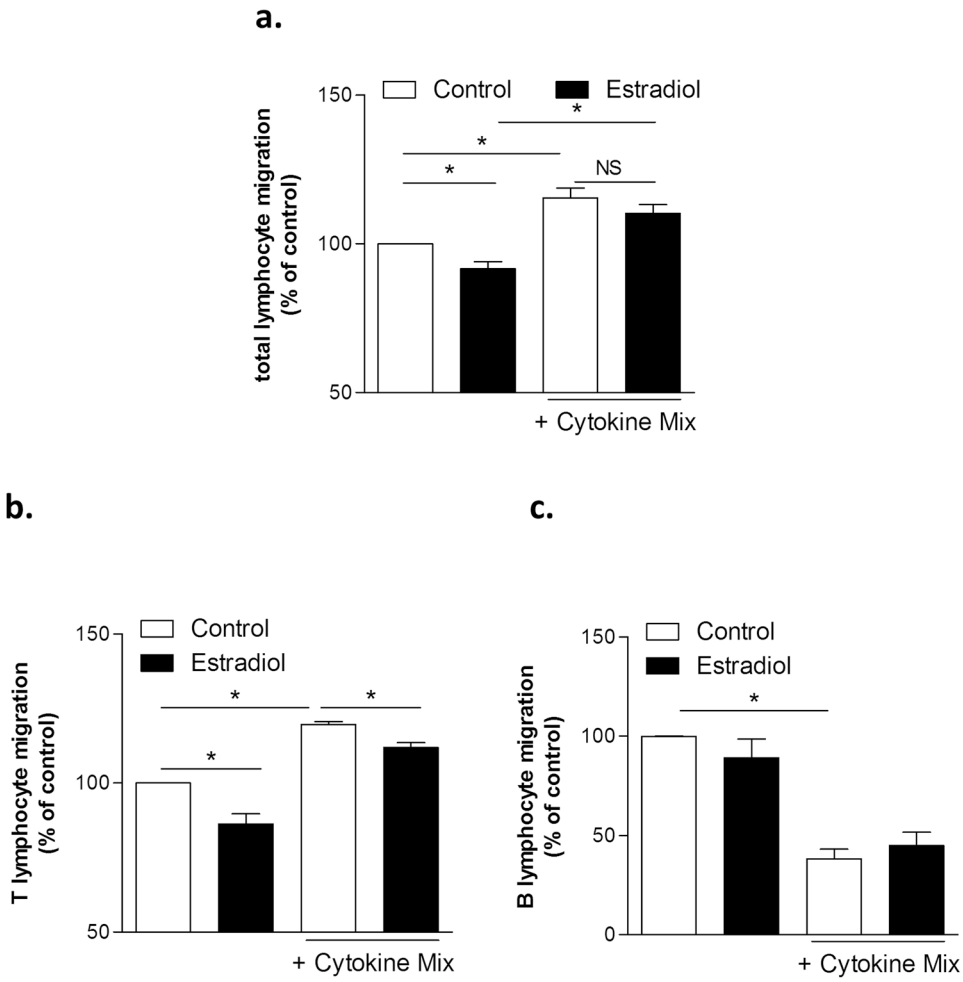

Figure 5. Analysis of Chemotactic properties of supernatants of human primary TECs treated with β-estradiol with or without a pro-inflammatory cytokine mix. Chemoattraction of PBL (**a**), T lymphocytes (**b**) and B lymphocytes (**c**) by supernatants of primary human TEC cultures treated with 17-β estradiol (10^{-8} M) with or without a mix of pro-inflammatory cytokines (IFN-γ, IL-1β, and TNF-α) for 24 hours. Results are expressed as the percentage of migrating cells through the well (±SEM). Each experiment was normalized to 100 for untreated cells. n = 4 to 6 different blood donors. P values were obtained using the Wilcoxon test.

Discussion

Numerous studies have demonstrated that females are more susceptible to autoimmune diseases. Two groups have recently cleared up this evidence highlighting a guilty contribution to sex hormone by their involvement in the regulation of AIRE, a molecule with a pivotal role in the mechanisms of central tolerance[19,39]. In addition, another transcription factor, vestigial-like family member 3 or VGLL3, has recently been identified as a regulator of inflammatory network that promotes female-biased autoimmunity[40]. Women have also a stronger response to infection, inflammation or vaccination due to an increased antibody production[41]. Truffault *et al*. have recently demonstrated that 80% of thymic hyperplastic MG patients are females, suggesting that female related features such as hormonal status may be taking into account for the development of such disease phenotype[3]. Although the fact that women may be more sensitive than men to autoimmunity is understandable, it remains inexplicable why they preferentially develop thymic hyperplasia in MG. Therefore, the aim of this study was to investigate the role of estrogens in the pathogenic mechanisms associated with AChR sensitization and thymic hyperplasia, by studying the expression of MG-associated molecules including the biological events involved in GC development such as chemokine expressions.

Estrogens and pathogenic mechanisms of MG. Estrogen display pro-inflammatory as well as anti-inflammatory effects through cytokine production regulation that depend on cell types, estrogen doses and the environment[42–46].

Estrogens are potent driver of regulatory T cells (Treg) by promoting their proliferation[47], by enhancing their suppressive activity[47] through the activation of the programmed cell death protein 1 (PD1) pathway[48] and by stimulating IL-10 secretion[49]. Our study confirmed the anti-inflammatory properties of estrogens and demonstrated that most of the molecules involved in the MG autoimmune response and the generation of GCs were down-regulated by estrogens. Interestingly, comparable estrogen effects have been shown in various human cell

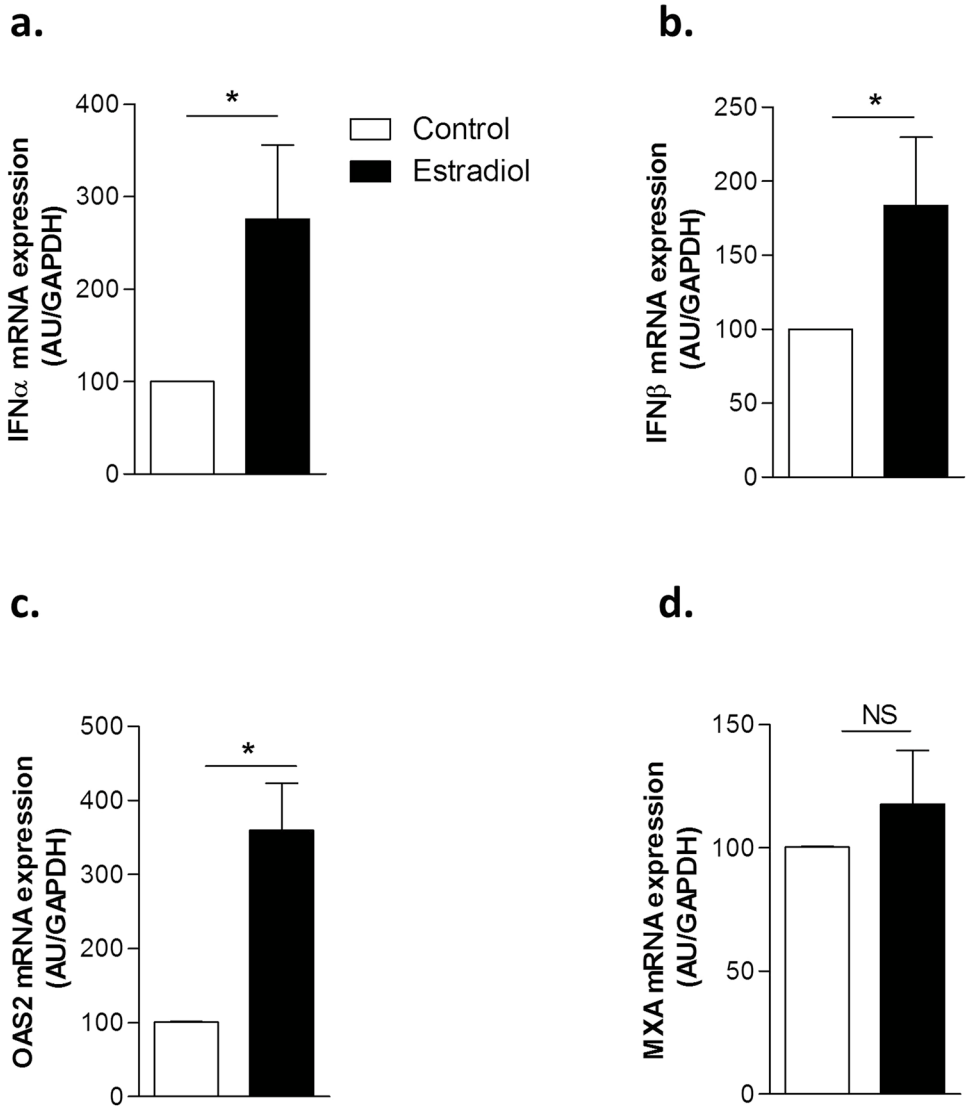

Figure 6. Modulation of interferon α, β, OAS2 and MXA gene expression in human primary cultured TECs by β-estradiol. Effects of 17-β estradiol (10^{-8}M) on IFNα (**a**), IFNβ (**b**), OAS2 (**c**) and MXA (**d**) mRNA expression in human primary TECs. Total mRNA were extracted from four different cultured human TECs treated for 24 h. P values were obtained using the Mann-Whitney test.

types among them human endometrial epithelial cells[50], vaginal cells[51] and intrahepatic biliary epithelial cells[52] for which β-17 estradiol inhibited HLA-DR expression at transcript and protein levels. One of the main TEC functions is to express tissue-specific antigens[53] such as α-AChR to set up the tolerance. This process of negative selection leads to the deletion of T-cells that strongly react with autoantigens presented by the medullary TECs through MHC class II[27]. We showed here that estrogens inhibited the expression of α-AChR and HLA-DR in TECs, suggesting that estrogens may alter the tolerization process, and favor the environment for an autoimmune response against α-AChR. Hence, these data suggest that estrogens in resting conditions create a "Yin/Yang" balance in the thymus by decreasing the expression of most chemokines that may reduce the autoimmune response but in same time alter the efficiency of the negative thymic selection.

Since estrogens display anti-inflammatory features[54], one could have expected that estrogens down-regulate the expression of the genes involved in the anti-AChR autoimmune response in MG patients. The facts are in contradiction with this hypothesis since MG thymus displays inflammation and germinal centers especially in females[3]. One possible explanation for this discrepancy is related to the finding that estrogens can promote IFN-I expression in TECs. Since type I interferon can stimulate its own production in an autocrine manner, and upregulate the expression of chemokines, it could explain the GC development in the thymus of females. These data corroborate previous studies that have shown in another cell types, a positive regulation control of estrogens on interferon type 1 and type 2 genes[37,55] and then on interferon-related gene levels. More, estrogens and IFN activate signaling pathways that work together to modulate estrogen- or IFN-sensitive genes[37]. Our study shows that expression of HLA-DR and α-AChR in human primary TECs was upregulated by inflammatory

cytokines, confirming previous studies[31], and similarly to what has been found in inflamed intestinal epithelial cells[56]. As recently suggested elevated expression in "inflamed" TECs of α-AChR favors, through an antigen cross-presentation by dendritic cells, the AChR autosensitization found in MG thymus[57]. Altogether, these observations tend to demonstrate that, in resting conditions, a low expression of α-AChR and HLA-DR by TECs induced by estrogens may result in a less efficient tolerance process that facilitates an increased women susceptibility to MG. However, once the pathology occurs, estrogens impact is restrained by the inflammatory molecules that control the immune response. Finally, the activation of IFN-I production by estrogens could interfere in the pathogenic processes.

ERE on gene promoter of molecules implicated in MG pathogenesis. To corroborate the *in vivo* results, we analyzed CXCL13, CCL21, CXCL12, α-AChR and HLA-DR human gene promoter by using a predictive tool for the transcription factor promoter region binding site, The Champion ChIP Transcription Factor Search Portal (Sabioscience) (Supplemental Fig. S6).

Analysis of promoter region of HLA-DR and α-AChR revealed the presence of regulatory transcription factor binding sites related to estrogens but also to NF-κB signaling pathways, which are highly activated in inflammatory conditions. In this context, although estradiol had a high downregulatory effect on HLA-DR and α-AChR subunits, in the presence of pro-inflammatory cytokines, the effects of estrogens were limited, indicating that the pro-inflammatory cytokines dominate for the expression of these genes. Indeed, the down-regulatory effects of estrogens were more striking, when the upregulatory effects of inflammation were limited. In the case of HLA-DR, cytokines increased the expression by 25 fold, while estrogens reduced it by a factor of 2.5. As a result, estrogens had no significant effect in the presence of inflammatory cytokines. This estrogen effect appeared to be, for HLA-DR, independent from ER-α, which may corroborate a possible activation of a ligand-independent genomic activation pathway through c-Jun N-terminal Kinase pathway[58] and or histone acetylation modifications[52]. By contrast, for α-AChR, estrogen regulation appeared to be clearly mediated through ER-α. The promoter region of CCL21, CXCL13 displayed ERE or XRE (for the Aryl Hydrocarbon Receptor), sites that required a recruitment of ER-α validating the involvement of estrogens in the control of the expression of these genes. The presence of ERE, in chemokine promoters, emphasizes the ability of estrogen alone to modulate cytokine and chemokine expressions. However, estradiol-induced changes in CCL21 expression were very limited (decrease factor of 1.4) in TECs even though the effects remained significant in the presence of cytokines (decrease factor of 1.6). Of note, in MG thymuses, it has been demonstrated that CCL21 increase is due to their production by lymphatic vessels corroborating the limited estrogens effect observed in TECs[59]. Altogether, our data demonstrated that the influence of estrogens was highly dependent upon the power of the inflammatory effect on a particular gene.

Estrogens transduction pathways and AIDs. One can speculate that if estrogens display duals operating rules, animal models with estrogen deficiency should display variable resistance to experimental MG. So far, data are not conclusive for the role of estrogen in the disease course. Delpy and colleagues have shown that estrogen administration aggravates EAMG symptoms in mice[60] while in rat[61] no estrogen effect was observed on the severity of muscle weakness. Moreover, female castration has no effect on the rat susceptibility to EAMG[62]. Recently, it has been demonstrated that ER-α KO mice display a similar susceptibility to EAMG compared to WT mice, and a preserved humoral and cellular immune responses to AChR except for the TNF-α response[63]. However, the classical EAMG mouse model mimics the muscle disease but does not reflects the human thymic features. A model that develops the thymic hyperplasic MG feature would be more helpful to validate or corroborate estrogens roles in this pathology.

Nevertheless, by using deficient mouse models for estrogens transduction pathways, several studies brought clues in the relationship between estrogens, autoimmunity and GC formation. Indeed, ER-α knockout mice exhibit immune complex-type glomerulonephritis, destruction of tubular cells and severe infiltration of B lymphocytes in the kidney[64] while ER-β deficient mice develop a bone marrow hyperplasia resembling myeloproliferative disease[65]. Even a defect in estrogen production, with aromatase KO mice, spontaneously leads to severe autoimmune exocrinopathy resembling Sjogren's syndrome characterized by signs of autoimmunity with lymphoproliferative phenotypes in bone marrow and spleen[66]. In these models, disequilibrium in estrogen transduction pathway appears to contribute to the apparition of autoimmune symptoms such as cell infiltrations or GC formation.

We can then suggest a similar mechanism in MG. Combined with the increased pro-inflammatory activity in the thymus[67], an abnormal decreased level of estrogens[68] in young MG female patients could be associated with a high chemokine and cytokine production leading to migration of B and activated T cells towards the thymus, and an efficient antigenic presentation could, in turn, leads to the formation of GCs. This hypothesis also fits with our previous work showing the increased expression of ER-α on thymic cells[23]. Indeed, high levels of estrogens downregulate the expression of ERs, so one can speculate that a defect of estrogens could be associated with higher ER expression and the development of B-cell hyperplasia, leading to the GC formation in the highly activated thymus of MG patients. Alternatively, estrogens at normal or high levels could indirectly affect thymic inflammation via a higher production of type I interferon, which influences the expression of molecules involved in GC development.

Conclusion. Our results highlight the subtle effect of estrogens. In resting conditions, estrogens have a dominant regulatory effect. However in an inflammatory milieu, the effects of estrogens were modulated: when cytokines produced a strong regulation, estrogens were not able to overcome it; however, in the absence or for low cytokine effect, estrogen did effectively affect the expression of molecules involved in autoimmune responses. Here, we demonstrated that equilibrium between estrogens and inflammatory cytokines could occur based on the

strength of their respective effect. Therefore, this suggests that the estrogen-induced low chemokine expression in woman thymuses is overpassed when stimuli activate inflammatory pathways such as IFN-I related one. However, estrogens can modulate IFN-I production. Consequently estrogens operated a twist to contribute to hyperplastic thymic in MG by sustaining the inflammatory pathway (Supplemental Fig. S7).

Materials and Methods

Human samples. Human thymic fragments (50–100 mg) were obtained from immunologically normal male and female patients (babies aged two days to 1-year-old, and adults aged 15 to 27 years old) undergoing corrective cardiovascular surgery at Marie Lannelongue Chirurgical Center (Le Plessis-Robinson, France). All tissue samples were fast frozen in liquid nitrogen within 30 minutes of their excision from patients for mRNA analysis or put in sterile RPMI medium for culture experiments.

C57BL/6 mice were purchased from Janvier Laboratory (Saint Berthevin, France). Aromatase knockout mice (ArKo) were generated by the disruption of the Cytochrome P450 19A1 gene (Cyp 19) and were kindly provided by Evan R. Simpson[66]. They were backcrossed under C57BL6 background. Mice were 6 to 10 weeks old ($n \geq 6$ per group). ERα−/− and ERβ−/− mice[69] backcrossed under C57BL/6 background (more than ten generations) were from Dr. Habert's mice colony (CEA, Fontenay-aux-Roses, France) under material transfer authorization #2010-036 of "Institut Clinique de la Souris", (Strasbourg, France). Male and female mice were 6 to 10 weeks old.

Primary cell cultures. Primary human thymic epithelial cell (TEC) cultures were established following the protocol previously described[70]. To avoid phenol hormone-like effects, RPMI phenol free was used. The culture medium was supplemented with 20% horse serum (Life Technologies, Invitrogen Corporation, Cergy-Pontoise, France), 0.2% Ultroser G (Life Technologies, Carlsbad, USA), two mmol/liter l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone. After 7–10 days, the cells were washed with phosphate-buffered saline (PBS) and collected by adding 0.075% trypsin (Life Technologies, Carlsbad, USA) and 0.16% EDTA for 10 min at 37 °C. Cells were then seeded and allowed to attach to the flask for 24 h before treatment in culture medium was supplemented with 5% horse serum.

Cells were treated for 24 hours with 17-β estradiol at 10^{-8} M (Sigma) in the presence or absence of a mix of cytokines (1 ng/mL recombinant human interleukin1β (IL-1β) (Sigma, Saint Quentin Fallavier, France), 10 ng/mL recombinant tumor necrosis factor-α (TNF-α), and 500 U/mL recombinant human interferon-γ (IFN-γ) (Genzyme, Cergy Saint Christophe, France) conditions previously described in Nancy *et al.*[23].

RNA extraction and reverse transcription. Thymuses were homogenized with the FastPrep FP120 instrument (Qbiogen, Illkirch, France). Total RNA was prepared from the thymus and TECs using the trizol RNA Isolation kit (Invitrogen, Cergy-Pontoise, France). The quality and concentration of RNA were analyzed with a NanoDrop ND-1000 spectrophotometer (LabTech, Palaiseau, France). RNA samples presenting a minimal ratio of 1.9 and 2 for respectively 260/280 and 260/230 were also controlled on a denaturing agarose gel. When the samples were degraded even partially, they were excluded. Total mRNA (1 μg) was reverse-transcribed using the SuperScript II RT kit (Invitrogen Cergy-Pontoise, France) according to the manufacturer's instructions.

Microarray experiments. Microarray experimentation procedure has previously been described by Le Panse *et al.*[9,19]. To minimize inter-individual variation, microarray experiments were performed with pools of RNA prepared with the equal amount of total RNA extracted from 4 thymuses of female donors aged 15 to 19 years old or from 3 thymuses of male donors aged 15 to 27 years old. The female and male RNA pools were co-hybridized respectively five and four times with a thymic reference composed of 10 thymuses of female babies aged one week to 1-year-old. All total RNAs were purified on Qiagen columns (Courtaboeuf, France) and their quality was assessed on an Agilent Bioanalyzer (Massy, France).

The experiments were performed with the "Human 1" cDNA arrays from Agilent (G4100A) according to the manufacturer's instructions by using 20 μg of total RNA.

Quantitative Real-Time PCR. Gene expression was evaluated by quantitative real-time PCR performed using the LightCycler apparatus (Roche Diagnostics, Meylan, France) as previously described by Dragin *et al.*[19]. The primers used are listed in Supplemental Table 2.

Each PCR was performed using the Fast-start DNA Master SYBR Green I kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions with the following conditions: initial denaturation at 95 °C for 10 min, then 40 cycles at 95 °C for 15 s, 60 °C for 14 s and 72 °C for 10 s, and a final fusion curve at 65 to 95 °C for 1 min.

Each cDNA sample was run at least in duplicate mRNAs were normalized to GAPDH. mRNA were expressed as arbitrary units and are the mean values (±SEM). For primary human TEC analysis, mRNA expression was normalized to 100 for untreated cells.

ELISA. The levels of CXCL13, CCL21, and CXCL12 were analyzed in TEC supernatants. Plates were coated overnight at 4 °C with 2.5 μg/ml of mouse anti-human CXCL13 antibody (MAB801) or chicken anti-human CCL21 antibody (AF336). Cell supernatants (1/100 dilution) or standards were incubated for 90 minutes at room temperature, and subsequently, 0.25 μg/ml of biotinylated anti-human IgGs and streptavidin-horseradish peroxidase were added.

Recombinant human CCL21 (366-6 C/CF) and CXCL13 (801-CX-025) were used as standards. Tetramethylbenzidine was used for color development, and plates were read at 450 nm using MRX reader DYNEX (Thermo Lab systems, Cergy-Pontoise, France). All antibodies were purchased from R&D systems (Lille, France).

Flow cytometry analyses. To analyze α-AChR subunit that is expressed intracellularly in TECs, cells were fixed and then permeabilized using IntraPrepTM Permeabilization reagents (Beckman-Coulter, Villepinte, France) according to the manufacturer's instructions. The permeabilized cells were then labeled with an anti-AChR antibody (clone mAB 35; Sigma, Saint Louis, USA). The staining was detected by FITC coupled-anti rat immunoglobulins (Valbiotech, Paris, France).

HLA-DR was analyzed in non-permeabilized TECs with a mouse anti-human HLA-DR conjugated to FITC (clone B8.12.2, Immunotech Marseille, France). In all flow cytometry analyses, the results show the median (±SEM) fluorescence intensity (MFI). For each experiment, the MFI was standardized to 100 for untreated cells.

Chemotaxis assays. TECs were treated for 24 h, and then supernatants were collected. Chemotaxis assay was performed in transwell plates (Costar/Dutcher, Issy-Les Moulineaux, France). TEC supernatants were placed in the lower wells while PBMCs were seeded in the upper wells. After five hours of incubation, cells were collected from both lower and upper wells and labeled with PE-coupled anti CD19 and FITC coupled anti-CD3-antibodies (DAKO Cytomation, Les Ulis, France). PBMCs were counted by flow cytometry assay calibrated using control microbead CaliBRITETM (BD Bioscience, Le Pont de Claix, France).

Promoter sequence extraction and detection of ER-binding sites. The gene promoter sequences were obtained from the UCSC Genome Bioinformatics Site. We then used SABiosciences Text Mining Application, a tool for retrieving human/mouse putative orthologous promoter regions.

Statistical analyses. Parametric or non-parametric test (Wilcoxon test for paired data, and Mann-Whitney test for unpaired data) were used to compare groups. Non-parametric tests were used thoroughly. However, because of the lack of power of these tests when the samples were too small ($n < 4$), in the few experiments with $n < 4$, the Student t test was used. The test is specified in the figure legend. Values are reported as Mean ± SEM. Statistical significance is recognized at $p < 0.05$. We used *GraphPad Prism 5* software to generate the graphs and to perform the statistical analyses. In all figures, the significance is displayed as stars, as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Ethics approval and consent to participate. The use of human tissue included in the present study was approved by the local ethics committee (CPP, Kremlin-Bicêtre, France: agreement No. 06–018; CCP Ile de France Paris 6, France agreement No. C09-36).

All animals were handled under the Sonia Berrih-Aknin authorization from the French Ministry of Agriculture (agreement no. 075–1792) and according to the Animal Care and Use of Laboratory Animal guidelines of the French Ministry of Agriculture.

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Author Contributions

N.D. and P.N. performed the experiments, analyzed the data and interpreted the results. J.V. performed some cell cultures experiments. R.R. provided human thymic tissues. R.L.P. provided helpful suggestions to design experiments. S.B.-A. was involved in all aspects of the study including: design, data analysis and interpretation of the results. N.D. and S.B.-A. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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RÉSUMÉ

La myasthénie grave (MG) est une maladie auto-immune chronique affectant les muscles due à des autoanticorps dirigés contre les récepteurs à l'acétylcholine (RACh). Le thymus est le site de production des anticorps pathogènes. Le thymus présente une inflammation caractérisée par une fonction défectueuse des cellules T régulatrices, une surexpression des cytokines IL-1β, IL-6, TGF-β1 et IL-21, de chimiokines et par la présence des centres germinatif ectopiques (eGC ; site de production des anticorps). Le cocktail de cytokines surexprimées dans le thymus favorise le développement des cellules T pro-inflammatoires Th17. Associé à ce cocktail, la cytokine IL-23 favorise l'émergence de cellules Th17 pathogéniques, impliquées dans la pathogenèse de diverses maladies auto-immunes.

Le premier objectif de ma thèse a été de déterminer les rôles potentiels de la voie IL-23/Th17 dans les évènements pathogéniques du thymus myasthénique et de caractériser les mécanismes thymiques impliqués dans le développement des cellules Th17. Mon deuxième objectif a été d'établir, in vivo, les effets thérapeutiques potentiels d'un anticorps bloquant l'IL-23 en utilisant deux modèles murins de MG.

Nous avons pu démontrer une surexpression de l'IL-23 par les CET myasthéniques ce qui dans l'environnement cytokinique du thymus MG favorise le développement de cellules Th17 pathogéniques et leur sécrétion accrue de l'interleukine 17. Nous avons montré que la dérégulation des voies de l'IFN-I dans le thymus myasthénique, stimule l'expression de l'IL-23 par les CET. De plus, les cellules Th17 opèrent un rétrocontrôle positif sur l'expression de l'IL-23 en stimulant via l'IL-17 les CET. Nous avons aussi observé un regroupement des cellules Th17 autour des eGCs et leur capacité à exprimer une protéine de stabilisation des eGC, la podoplanine.

Afin de déterminer les effets thérapeutiques potentiels du blocage de l'IL-23 sur l'inflammation thymique, nous avons utilisé le modèle de greffes sous cutanées de thymus MG dans les souris immunodéficientes (NSG-MG) décrit par Sudres et al. L'anti-IL-23 induit une réduction du pourcentage des cellules Th17 thymiques et circulantes ainsi que l'expression thymiques de l'IL-17 et des protéines associées aux eGC (AID et podoplanine). De plus, le traitement induit une diminution des signes cliniques de myasthénie. Dans le modèle murin classique de la myasthénie (EAMG), qui consiste à immuniser avec du RACh, et qui permet l'étude des effets des auto-anticorps sur le muscle, une amélioration des signes cliniques consécutive au traitement anti-Il-23 a été observée. Dans ce modèle EAMG, une diminution du taux circulant des auto-anticorps et des marqueurs inflammatoires dans le muscle tibialis anterior (TA) (IL-6 et IL-17) est mesurée. De plus, le traitement semble induire une activation de la régénération musculaire et une diminution de l'activation de cellules souches musculaires (cellules satellites).

En conclusion, l'ensemble de mes résultats de thèse ont permis de montrer que l'IL-23 est impliquée dans les processus inflammatoires et physio-pathogéniques présents dans le thymus myasthénique. Son blocage avec des anticorps monoclonaux dans les deux principaux modèles murins de myasthénie auto-immune a abouti au contrôle de l'inflammation thymique, la réduction de la production d'autoanticorps et une amélioration des signes cliniques de la pathologie. Si ce résultat encourageant et prometteur est validé sur d'autres modèles de MG, il permettra de proposer une nouvelle stratégie thérapeutique avec un repositionnement de molécules utilisées pour les patients atteints de Psoriasis (guselkumab, anti-IL-23p19) et une possibilité de réduire le recours à la thymectomie pour les patients atteints de myasthénie auto-immune $RACh^{+}$.