



Characterization of thymic hyperplasia associated with autoimmune Myasthenia Gravis: role of the chemokines CXCL12 and CXCL13

Julia Miriam Weiss

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PhD thesis

Characterization of thymic hyperplasia associated with autoimmune Myasthenia Gravis: Role of the chemokines CXCL12 and CXCL13

Presented by

Julia Miriam WEISS

Supervisor

Dr Rozen LE PANSE

Thesis director

Dr Sonia BERRIH-AKNIN

November 28, 2011

JURY

Dr Karl BALABANIAN

Dr Sonia BERRIH-AKNIN

Dr Christophe COMBADIÈRE

Pr Bruno EYMARD

Dr Rozen LE PANSE

Pr Xavier MARIETTE

In the memory of my mother and hers.

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ABSTRACT

Autoimmune myasthenia gravis (MG) is a muscular disease mediated by autoantibodies, mainly directed against the acetylcholine receptor (AChR). The pathogenic antibodies are especially produced in the thymus, which is often characterized by a hyperplasia with germinal centers. Recent studies demonstrated the overexpression of chemokines and the abnormal development of high endothelial venules (HEV) in the MG thymus. **The aim of my thesis was to better understand the mechanisms that lead to thymic hyperplasia in MG by analyzing the role of chemokines in peripheral cell recruitment.**

We demonstrated that the number of HEVs correlated with the degree of hyperplasia suggesting a direct link between HEVs and peripheral cell recruitment. To define its mechanism of action, we examined which chemokines were expressed on thymic HEVs. We uniquely detected SDF-1 and observed that B cells, myeloid dendritic cells (mDCs), plasmacytoid DCs and monocytes/macrophages that expressed the SDF-1 receptor CXCR4 localized inside and around thymic HEV. In parallel we observed a decreased CXCR4 expression and a decreased number of mDCs and also monocytes in the periphery suggesting their recruitment to the MG thymus.

As the MG thymus was recently characterized by the overexpression of CXCL13 in thymic epithelial cells (TECs), we investigated its contribution to thymic hyperplasia. We therefore generated a transgenic mouse model overexpressing in medullary TECs CXCL13 under the control of keratin 5. We demonstrated that transgenic K5-CXCL13 mice specifically overexpressed CXCL13 in the thymus, while no other tested chemokines were upregulated. Preliminary results showed that elevated levels of CXCL13 resulted in an increased number of B cells in the thymus of transgenic mice, which localized preferentially in loose aggregates in medullary areas. We are presently investigating if immunization with purified AChR induces experimental MG with thymic hyperplasia in these mice. Myasthenic mice with a hyperplastic thymus could present a new animal model for MG with a phenotype that is closer to the human disease than the current MG model.

As the hyperplastic MG thymus displays the hallmarks of a viral signature, we investigated the effect of pathogen-associated molecules on thymic changes associated with MG. We demonstrated that dsRNA signaling induced by Poly(I:C) specifically triggers the overexpression of α -AChR in human TECs through the release of IFN-I. We also observed that IFN-I was able to upregulate CXCL13 and CCL21, similarly to what is observed in the MG thymus. In addition, Poly(I:C) injections in wildtype mice, but not in IFN-I receptor KO mice, specifically increase thymic expression of α -AChR and, in parallel, CXCL13 and CCL21 expression. In periphery, Poly(I:C) even induced an anti-AChR autoimmune response characterized by a significant production of serum anti-AChR antibodies and a specific proliferation of B cells.

Overall the results obtained in the course of my PhD showed that the abnormal development of SDF-1-expressing HEVs and the CXCL13 overexpression play a central role in the recruitment of peripheral cells to the MG thymus. Once these cells have arrived in the inflammatory environment, which is characteristic for MG, they could develop an autoimmune reaction against AChR. New therapeutic molecules that control chemokine expression and angiogenic processes could diminish the development of thymic hyperplasia and avoid thymectomy or the use of corticoids.

ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AChR	acetylcholine receptor
AIDS	acquired immune deficiency syndrome
AIP4	atrophin-1-interacting protein 4
AIRE	autoimmune regulator
APC	antigen presenting cell
cDCs	conventional dendritic cell
CIA	collagen-induced arthritis
CNS	central nervous system
cTEC	cortical thymic epithelial cell
DAG	diacylglycerol
DARC	duffy antigen receptor for chemokines
DC	dendritic cell
DN	double negative
DP	double positive
EAE	experimental autoimmune encephalomyelitis
EAMG	experimental autoimmune myasthenia gravis
EGFP	enhanced green fluorescent protein
EpCAM	epithelial cell adhesion molecule
FDC	follicular dendritic cell
GC	germinal center
HEV	high endothelial venule
ICAM1	intra cellular adhesion molecule 1
IFN	interferon
IFNAR	IFN-I receptor
Ig	immunoglobulin

IL	interleukin
IP3	inositol triphosphate
IRF	interferon regulatory factor
IvIg	intravenous immunoglobulin
K5	keratin 5
L-selectin	lymphocyte-selectin
LFA1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharides
LRP	low-density lipoprotein receptor-related protein
LUCIP	luciferase-reporter immunoprecipitation
mAChR	muscarinic acetylcholine receptor
MADCAM1	mucosal vascular addressin cell adhesion molecule1
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation-associated gene 5
MG	myasthenia gravis
MGFA	myasthenia gravis foundation of America
MHC	major histocompatibility complex
MIR	main immunogenic region
MS	multiple sclerosis
MSC	mesenchymal stem cell
mTEC	medullary thymic epithelial cell
MuSK	muscle specific kinase
nAChR	nicotinic acetylcholine receptor
NMJ	neuromuscular junction
PBMC	peripheral blood mononuclear cells
PNAd	peripheral-node addressin
pDC	plasmacytoid dendritic cell

PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLP	proteolipid protein
Poly(I:C)	polyinosine-polycytidylic acid
Plt	paucity of lymph node T cells
PSGL1	p-selectin glycoprotein ligand-1
PVS	perivascular space
RA	rheumatoid arthritis
RIA	radioimmunoassay
RIG-I	retinoic acid-inducible gene I
S1P	sphingosine-1-phosphate
SLO	secondary lymphoid organ
SP	single positive
T-AChR	torpedo acetylcholine receptor
TCR	T-cell receptor
TEC	thymic epithelial cell
Tg	transgenic
TLR	toll-like receptor
Treg	regulatory T cell
TSA	tissue-specific antigen
TSLP	thymic stromal lymphopoietin
VCAM1	vascular cell adhesion protein-1
VLA1	very late antigen4
WT	wildtype

INDEX

I. STATE OF THE ART	9
1. MYASTHENIA GRAVIS	10
1.1 GENERALITIES.....	10
1.1.1 HISTORY	10
1.1.2 EPIDEMIOLOGY	12
1.1.3 SYMPTOMS AND DIAGNOSIS	12
1.2 PATHOPHYSIOLOGICAL MECHANISMS IN MG.....	14
1.2.1 THE NEUROMUSCULAR JUNCTION UNDER PHYSIOLOGICAL CONDITIONS	14
1.2.2 PATHOGENIC MECHANISMS IN MG	16
1.2.3 GENETIC PREDISPOSITION	19
1.2.4 TREATMENTS.....	20
1.3 EXPERIMENTAL AUTOIMMUNE MG	22
1.4 NEW POTENTIAL THERAPIES	24
2. THYMUS	26
2.1 STRUCTURE AND FUNCTION	26
2.1.1 GENERALITIES.....	26
2.1.2 THYMIC CELLS	28
2.1.3 T CELLS: MATURATION BY NEGATIVE AND POSITIVE SELECTION.....	31
2.2 PATHOLOGICAL ROLE.....	35
2.2.1 THYMIC DISORDERS	35
2.2.2 THYMOMA-ASSOCIATED MG	36
2.2.3 THE HYPERPLASTIC THYMUS IN MG.....	38
3. CHEMOKINES.....	44
3.1 GENERAL PROPERTIES.....	44
3.1.1 STRUCUTRE AND CLASSIFICATION	44
3.1.2 CHEMOKINE RECEPTOR SIGNALING	46
3.2 PHYSIOLOGICAL ROLE OF CHEMOKINES	48

3.2.1 LYMPHOCYTE HOMING	49
3.2.2 THYMOPOIESIS.....	53
3.3 PATHOLOGICAL ROLE OF CHEMOKINES.....	54
3.3.2 CHEMOKINES IN MG THYMUS	55
II. AIM OF THE STUDY	58
III. RESULTS.....	61
Article 1	62
Article 2	97
Article 3	126
Article 4	162
IV. GENERAL CONCLUSION.....	175
V. PERSPECTIVES.....	180
VI. REFERENCES.....	182

I. STATE OF THE ART

1. MYASTHENIA GRAVIS

Autoimmune Myasthenia Gravis (MG) is a rare neuromuscular disorder, which is characterized by muscle weakness caused by a defective transmission of nerve impulses to muscles. The defect is mediated by autoantibodies against components of the neuromuscular junction (NMJ) on the postsynaptic membrane of striated skeletal muscles. Autoimmune MG must be distinguished from congenital MG, which exhibits similar symptoms due to genetic mutations and not due to immunopathogenic dysregulation. In the following study, the term “MG” refers exclusively to autoimmune MG.

1.1 GENERALITIES

1.1.1 HISTORY

The first case report of MG occurred in 1672, when the English physician Willis described a woman who “*for some time can speak freely and readily enough, but after she has spoke long, or hastily, or eagerly, she is not able to speak a word*” [1]. At the end of the 19th century, Erb and Goldflam established the first description of clinical symptoms attributed to MG: frequent ptosis with diplopia, dysphagia, weakness of the neck, and the course of remissions and relapses [2],[3]. Initially, MG was therefore referred to as Erb-Goldflam disease until 1899, when Jolly, who suggested a neuromuscular inhibitor in the circulation of patients, introduced the name “Myasthenia Gravis” composed of myasthenia, Greek for weakness, and gravis, Latin for severe [4].

Until 1885, MG had been only described in England, Germany and Austria. Inspired by the papers of Erb, Goldflam and Jolly, reports accumulated also in other countries including France, Italy and the Unites States [5]. In 1901, Lacquer and Weigert described for the very first time the finding of a thymic tumor in a myasthenic patient, however, no further attention was given to this observation [6].

In the following years, a better understanding of the neuromuscular transmission led to progress in understanding the pathogenic mechanisms in MG. In 1904, Elliot suggested that a chemical substance, liberated at the nerve endings, could initiate contraction in muscle fibers [7]. About twenty years later, Loewi and Dale identified this substance as Acetylcholine (ACh) and reported that its activity was

limited by cholinesterase [8]. Based on this knowledge, physiologists and pharmacologists tried to identify the inhibiting factor that caused the defect in the neuromuscular transmission in MG.

In parallel, remarkable progress was made concerning the treatment of MG. Edgeworth, a researcher and MG patient herself, was taking ephedrine for chronic sinusitis and discovered that it mediated a considerable improvement of her strength [9]. A few years later, in 1934, Denny-Brown and Walker realized that curare-poisoning lead to the same symptoms than MG and tested prostigmine, a synthesized antidote for curare-poisoning, with striking temporary improvement and prolongation of life expectancy for MG patients [10].

In 1939, 38 years after the first mentioning of an association between a thymic tumor and MG, Blalock discovered the beneficial effect of thymectomy and the presence of numerous germinal centers (GC) in the thymus of MG patients [11]. In 1959/60, Simpson and Nastuck proposed that MG was mediated by an autoimmune response indicated by (1) frequent association with other autoimmune diseases, (2) presence of transient MG symptoms in 10-15% of newborns from myasthenic mothers (neonatal MG), (3) inflammatory infiltrates in muscle and pathological changes in thymus, (4) beneficial effect of immunosuppressors, (5) effect of patients' sera on muscle contraction [12]. They hypothesized that antibodies in patients could bind to the receptor of ACh (AChR) on muscles and would thus block the neuromuscular transmission leading to MG syndrome.

In 1973, Patrick and Lindstrom observed that rabbits, immunized with AChR purified from the electric organs of the torpedo fish, developed MG-like symptoms [13] and had thus created the first animal model for MG. A few years later, Lindstrom developed a radio immune assay (RIA) that proved the presence of anti-AChR antibodies in the serum of 85% of MG patients [14]. The key role of these antibodies in disease development was demonstrated by the emergence of MG symptoms in animals transferred with purified immunoglobulins G (IgGs) from MG patients and by the degradation of AChR on cultured muscle cells after incubation with patient's IgGs [15]. Numerous studies have ever since confirmed the autoimmune reaction against muscle AChR and have identified other antibody targets in MG, in particularly the muscle specific kinase (MuSK) and only very recently the low-

density lipoprotein receptor-related protein 4 (Lrp4) [16],[17],[18]. Both Musk and Lrp4 are required for the clustering of AChR.

Since the first description of MG in the second half of the 17th century, enormous progress has been made concerning, diagnosis, therapy and understanding of MG physiopathology. Modern treatments are rather successful and patients have a normal life expectancy; nevertheless, some mysteries still remain: the lack of a correlation between disease severity and anti-AChR antibody level, the role of the thymus in patients without anti-AChR antibodies, the antigenic target(s) in patients without defined auto-antibodies, the female predominance, but most of all, the triggering events that induce the development of MG.

1.1.2 EPIDEMIOLOGY

Although MG is a rare disease, its prevalence rate has increased over time, most likely due to improvements in diagnosis [19]. MG occurs in any race, at any age and in either gender. The incidence rate in Europe ranges between 4 and 18 cases per year per one million inhabitants. Its prevalence lies between 70 and 163 cases per one million people [20]. Concerning the age of onset, there appears to be two incidence peaks with the first peak at the age of 20-40 years and a second peak at the age of 60-70 years. In patients younger than 40 years old, women predominate (70%). After the age of 50, new cases of MG are slightly more common in men (60%) [19]. The incidence rate apparently declines after the age of 70, however, using AChR antibodies as a diagnostic tool, a study in the UK showed that MG was considerably underdiagnosed in people older than 75 [21].

The events triggering the onset of MG are undefined. In a recent survey carried out in Norway, patients indicated pregnancy, stress, childbirth, operation, medical treatment or vaccinations as factors related to their onset [22].

1.1.3 SYMPTOMS AND DIAGNOSIS

The characteristic feature of MG is fatigability of the voluntary muscles. During periods of activity, the muscles become gradually weaker and improve after periods of rest. In more than 50% of all cases,

the initial symptoms are dropping eyelids (ptosis) and double vision (diplopia). Only 15% remain confined to eye muscles (so-called ocular MG; mostly men >40 years), the rest develops a generalized muscle weakness affecting muscles involved in chewing, swallowing and talking, muscles in the neck, limb or trunk muscles [23]. The weakness usually progresses in craniocaudal direction, which means from ocular, to facial, to lower bulbar, to truncal, to limb muscles. In severe cases, breathing may be so weak that patients need a ventilator [24]. Patients with anti-MuSK antibodies have primarily facio-pharyngeal symptoms or weakness of neck and respiratory muscles, while they suffer rather rarely from ocular myasthenia [25]. In most patients, symptoms vary from day to day and from hour to hour, with the tendency to worsen towards the end of the day.

Certain conditions can trigger or impair the muscle fatigability such as hyperthermia, ambient temperature, menstruation, pregnancy, infections, emotional stress and certain drugs including some antibiotics (e.g streptomycin), anti-rheumatics (chloroquine), NMJ blockers, anti-malarials, botulinum toxin, or beta blockers. In certain cases, myasthenic symptoms may be induced by bone marrow transplantation, treatment with D-penicillamine and administration of interferon (IFN)- α . [23]. Due to its fluctuating character and common features with other disorders, MG is still difficult to diagnose. Typically, the diagnosis is based on 1) pharmacological test by injection of anticholinesterase (AChE) drugs to elicit a rapid improvement of strength, 2) electrophysiological tests with repetitive nerve stimulation to prove a postsynaptic neuromuscular junction disorder and 3) serological tests to detect AChR or MuSK antibodies [26].

The MG foundation of America (MGFA) classification was established to allow an estimation of disease severity for the clinical follow-up and evaluation of treatment efficacy [27]. Within this classification, Class I refers to ocular weakness, Class II-IV correspond to mild, moderate or severe muscle weakness and are further subdivided in subclass A or B depending on the affected muscle, while Class V is used to describe patients that require intubation and mechanical ventilation (table1).

Classification	General description	Subclass A	Subclass B
Class I	Ocular muscle weakness only		
Class II	Mild muscle weakness	Affects primarily limb and axial muscle	Affects primarily bulbar and respiratory muscle
Class III	Moderate muscle weakness	Affects primarily limb and axial muscle	Affects primarily bulbar and respiratory muscle
Class IV	Severe muscle weakness	Affects primarily limb and axial muscle	Affects primarily bulbar and respiratory muscle
Class V	Intubation		

Table 1: MGFA classification for disease severity [27]

MG is often accompanied by other autoimmune diseases, the most frequent ones are thyroid disorders, such as hyperthyroidism, hypothyroidism or goiter, but also rheumatoid arthritis, pernicious anaemia, systemic lupus erythematosus, sarcoidis, Sjogren's syndrom, polymyositis, ulcerative colitis and pemphigus [24].

1.2 PATHOPHYSIOLOGICAL MECHANISMS IN MG

1.2.1 THE NEUROMUSCULAR JUNCTION UNDER PHYSIOLOGICAL CONDITIONS

The NMJ is the synapse, where the terminal axon of a motor neuron encounters the muscle. Its primary task is to convert a weak nerve impulse into a muscle depolarization resulting in a muscle contraction. Each axon divides into branches that innervate several muscle fibers. These branches are again subdivided into many presynaptic buttons, which contain presynaptic vesicles loaded with ACh [26]. ACh is synthesized from acetyl Coenzyme A and choline by the enzymatic action of choline transferase and packaged into vesicles, each containing around 8000-13000 ACh molecules (=quanta). When a nerve impulse arrives, the presynaptic nerve terminal is depolarized and an influx of calcium is produced via voltage-gated calcium channels. Subsequently, the ACh-containing vesicles fuse with the presynaptic nerve terminal membrane. As a result, ACh gets released and can interact with the nicotinic AChR (nAChR) on the muscle.

The nAChR is a transmembrane pentameric protein complex arranged around a central channel (figure 1, left). nAChRs are located in clusters on the muscle endplate of the postsynaptic membrane. In fetal or denervated muscles, nAChRs consist of two identical α -subunits and three different subunits β , δ and γ . In humans, the γ -subunit is gradually replaced by the ϵ -subunit upon 30 weeks of gestation, however the γ isoform continues to be expressed in adults in extraocular muscles, thymic myoid cells and, at low levels, thymic epithelial cells (TECs) [28],[29].

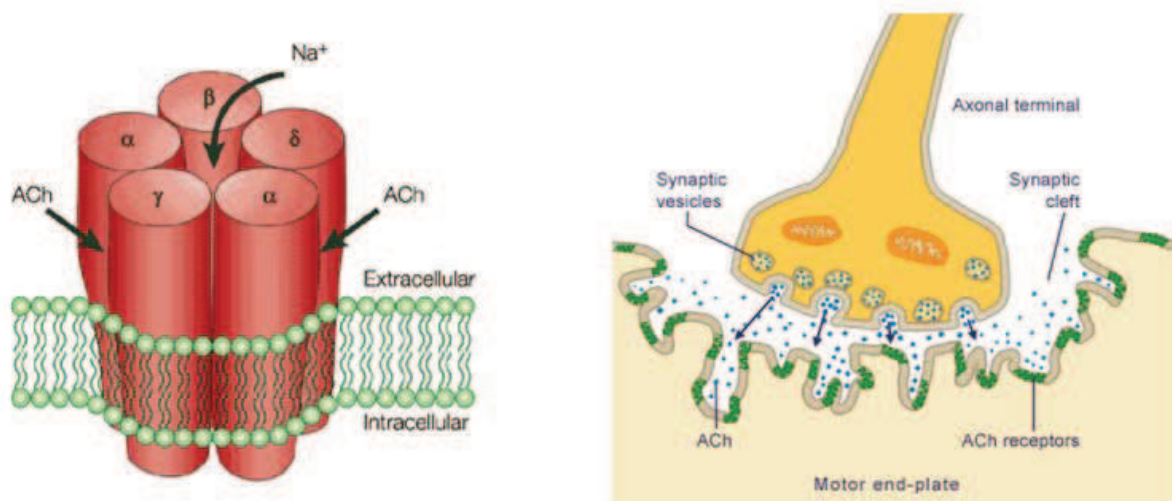


Figure 1: Schematic structure of the nAChR (left) and the NMJ (right), adapted from Karlin *et al.*, Nature Reviews Neuroscience 2002 [30] and http://alexandria.healthlibrary.ca/documents/notes/bom/unit_2/L-08%20Regulation%20of%20Muscle%20Contraction%20and%20Force%20Output.xml

In a resting state the ion-channel of the nAChR is closed and opens when both α -subunits are occupied by ACh. This allows the influx of cations into the muscle cell, which leads to membrane depolarization called the endplate potential. If the endplate potential reaches a certain threshold, voltage-sensitive sodium channels localized at the base of synaptic folds at the muscle endplate open. Under physiological conditions, the excitation of this potential is guaranteed by an excess of released ACh and an abundance of nAChR (“safety factor”). ACh has a very short half-life of two minutes and binds only transiently to its receptor, before it either diffuses or is hydrolyzed by AChE present in the synaptic cleft. These events terminate the neuromuscular transmission [31] (figure 1, right). The degradation and the synthesis of nAChR are balanced with a half-life of 6-13 days [32].

The nAChR must be distinguished from muscarinic AChRs (mAChR), which are not ion channels but members of the superfamily of G-protein-coupled receptors. They are mainly acting in the central nervous system [33]. While both kinds of receptors are capable of binding ACh, the nAChR can in addition bind to nicotine and the mAChR to muscarine, hence their nomination.

1.2.2 PATHOGENIC MECHANISMS IN MG

The changes of the NMJ in MG include a widened synaptic cleft, reduced number of AChRs and simplification of the post-synaptic cleft (figure 2). These abnormalities are caused by an autoimmune attack on the postsynaptic membrane, which leads to a reduction of the “safety factor”. According to the specificity of the autoantibodies involved in the autoimmune attack, MG can be subdivided in 3 categories: AChR MG, MuSK MG and seronegative MG.

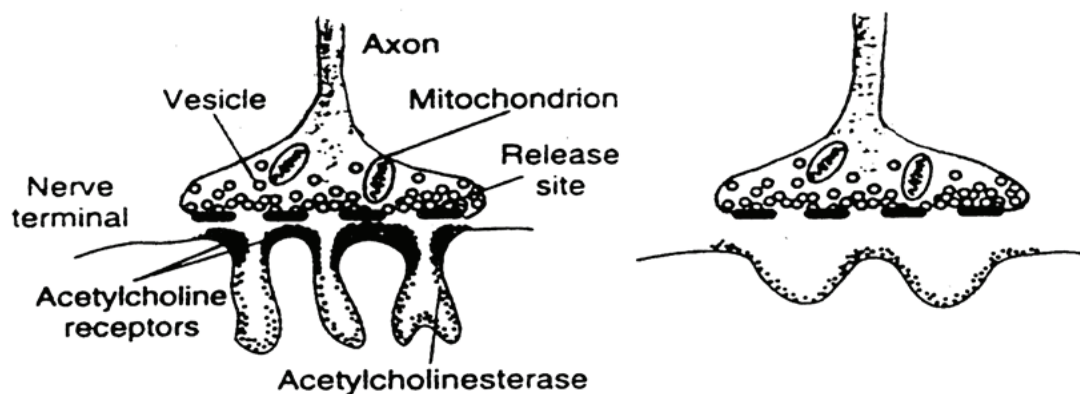


Figure 2: NMJ of a healthy (left) and an MG (right) muscle
(adapted from <http://totw.anaesthesiologists.org/2008/12/14/anaesthesia-and-myasthenia-gravis-122/>)

AChR MG

In 85% of MG patients the autoreactive antibodies are directed against the AChR located at the postsynaptic muscle endplate membrane. The antibodies in one individual are composed of different subclasses of IgG antibodies and target preferentially a region on the extracellular side of the α -subunit of the AChR, called MIR for main immunogenic region [24]. Nevertheless, anti-AChR antibodies

against other parts of the α -subunit as well as other AChR subunits can be detected in MG patients [34], [35].

The binding of antibodies to the AChR results in so far three identified mechanisms: 1) accelerated degradation and internalization of AChR crosslinked by autoantibodies (=antigenic modulation), 2) blockade of the ACh binding site, 3) binding and activation of complement at the NMJ [36],[37]. Several studies indicate that the complement activation, which results in lysis of the post-synaptic muscle membrane, is the primary cause for AChR loss [38],[39]. Experiments in rats showed that blocking or depletion of complement protects animals from experimental MG [38]. Besides, interleukine-12 (IL-12) deficient mice, which poorly synthesize complement-binding antibodies, develop minimal MG symptoms after AChR immunization despite strong production of anti-AChR antibodies that were found at the NMJ [40].

The level of anti-AChR antibody does not correlate with the clinical severity of MG [40]; even though titer variations in an individual patient can correlate roughly with clinical changes [23]. AChR MG patients with a thymoma have also antibodies against titin and in 50% of the case also antibodies against ryanodine receptor, both are components of the striated muscles [41],[42]. AChR MG, especially in early onset patients, has a female predominance with a ratio of 1:3 between men and women [43].

MuSK MG

A second category of MG patients, corresponding to less than 5% of all patients, is defined by the presence of auto-antibodies against MuSK. MuSK is a tyrosine kinase and plays a crucial role in the clustering of AChRs and other postsynaptic components at the NMJ. Experiments in MuSK knockout mice demonstrated that interruption of this process leads to impaired transmission at the neuromuscular junction [44]. MuSK antibodies are partially IgG1 but predominantly IgG4 and unlike AChR MG patients, antibody titers correlate with disease severity [45]. MuSK MG patients exhibit roughly the same clinical signs than AChR MG patients, but their weakness is typically more severe

and frequent crises occur early in the disease course. In terms of therapy, MuSK patients differ from AChR patients by a poor responsiveness to acetylcholinesterase inhibitors and a need for more aggressive immunosuppressive treatments with fewer patients achieving remission [25]. 85% of MuSK patients are women with disease onset typically in the fourth decade [46].

Seronegative MG

Seronegative MG patients are patients for which no serum autoantibodies against AChR or MuSK can be detected by routine tests. Clinical reports on 100-150 seronegative patients are available in the literature showing great heterogeneity for this group of MG patients [47]. The age of onset is quite variable ranging from 1-83 years without any obvious predominance in women. Many seronegative patients have ocular and limb involvement, while bulbar and respiratory muscles are rarely affected and disease severity is rather mild compared to patients with AChR- or MuSK-antibodies. The clinical features of seronegative MG globally resemble those of patients with anti-AChR antibodies and the treatments proposed to seronegative MG patients are the same as those for AChR-MG.

Previously, the hypothesis was raised that 'seronegative' patients may have antibodies against AChR, which could not be detected by routine tests due to low serum levels or low receptor affinity. By testing the binding of antibodies from seronegative patients to dense aggregates of AChR on an *in vitro* cell line, Leite *et al.* demonstrated that two third of seronegative patients had indeed low affinity anti-AChR antibodies in their serum [48].

Very recently, Lrp4, a molecule involved in the clustering of the AChR, was identified as a novel target for autoantibodies in a proportion of seronegative MG patients [17],[18]. Hihuchi *et al.* established a luciferase-reporter immunoprecipitation (LUCIP) assay, in which the strongly luminescent *Gaussia* luciferase is coupled to Lrp4 to detect anti-Lrp4 antibodies binding. These antibodies belonged to the complement activating IgG1 subclass and blocked interaction between Lrp4 and its ligand agrin. In this study, Lrp4 antibodies were found in 3 out of 28 MuSK patients. Another

study of Pevzner *et al.* showed that 50% of seronegative patients had sera that specifically bound to cells transfected with human LRP4 [18].

1.2.3 GENETIC PREDISPOSITION

While congenital MG is caused by mutations in genes coding for components of the neuromuscular junction, the genetic contribution to autoimmune MG is less obvious. Only few reports exist about families with multiple cases of MG, but an increased risk of MG or other autoimmune diseases among the relatives of MG patients support the idea of a genetic predisposing background favoring the susceptibility to MG [49],[50],[51],[52]. Studies comparing the genome between controls and non-related MG patients allowed to identify gene variations that are associated with MG. Among the genetic factors analyzed, the most striking one was the major histocompatibility complex (MHC) [53]. MHC has been associated with MG in several studies, in different cohorts and shows the largest influence with multiple effects. In particular HLA-DR3 and B8 alleles were observed to be associated in Caucasian patients with thymic hyperplasia [54-56]. Different MHC variations were detected in Asian patients and in late-onset European MG patients suggesting that the heterogeneity of MHC genes reflects the biological heterogeneity of MG patients [57-59]. Other genes associated with MG include those encoding for *IL-10*, *IL-1 β* , *TNF- α/β* , *IFN- γ* , *CTLA4* (Cytotoxic T-Lymphocyte Antigen 4) and *PTPN22* (cellular tyrosine phosphatase 22) [60]. Recently, a promoter variant controlling the transcription of the *CHRNA1* gene, which encodes the α -subunit of the AChR, was found to be more common in early-onset MG patients [61]. Further investigations showed that a single nucleotide polymorphism converting an adenine to a guanine in the promoter sequence disrupted the binding site for IFN regulatory factor 8 (IRF8). In parallel, the promoter binding of the transcription regulator AIRE, which is involved in the thymic expression of multiple peripheral tissue specific antigens, was impaired leading to a two-fold decreased level of *CHRNA1* mRNA in TECs. The reduced expression level was proposed to alter the tolerance towards α -AChR and thus to increase the risk to develop MG. At present, a study including more than 1000 patients and controls is performed in order to identify new genetic and epigenetic risk factors in MG.

1.2.4 TREATMENTS

While the mortality rate of MG was up to 30% in the first half of the 20th century, the prognosis for patients has improved drastically and nowadays they have a normal life expectancy due to advances in treatments.

Acetylcholine esterase inhibitor

By blocking AChE, AChE inhibitors prolong the half-life of ACh and thus the probability to interact with the AChR. Typically, AChE inhibitors are the initial drug used in MG treatment and may be the only drug applied in patients with mild disease. Pyostigmine is the classical and most often applied AChE inhibitor. Its major advantage is a rapid effect that lasts about four hours [62]. The beneficial effect tends to decline during continuous treatment. The administration of this drug risks several side effects and therefore requires a fine tuning and a careful dosing.

Corticosteroids

Treatment with corticosteroids, such as prednisolone, is applied in patients with moderate or severe MG or in mild cases that do not respond to AChE inhibitors. Their precise mechanism of action is unknown, but they have numerous effects on the immune system including a decrease of autoantibody production. Administration of steroids bears the risk of serious side effects. In long-term treatment, patients therefore start with a high-dose daily regimen and gradually lower doses, which are then combined with other immunosuppressive drugs. It may require several months or years to determine the minimal efficient doses.

Immunosuppressors

The most common immunosuppressor are azathioprine, cyclosporine and mycophenolate mofetil [63]. These molecules act by blocking nucleotide synthesis and lymphocyte proliferation and can be used in long-term treatment [24],[26]. However, strong side effects and high costs, respectively, make their administration less attractive.

Recently a chimeric, monoclonal antibody against B-cell marker CD20, rituximab, was introduced as a treatment for MG [64]. It reduces the number of circulating B cells, its precise working mechanism, however, is not known, yet. In MG patients resistant to other treatments, administration of rituximab was well tolerated and noticeably successful with no therapy-associated side-effects being observed [65].

In a pilot trial with the TNF- α blocker Etanercept, 70% of corticosteroid-dependent MG patients showed improved muscle strength and lowered corticosteroid requirement [66]. However, its administration must be handled with precaution, as in some MG patients disease severity worsened and MG symptoms occurred in a few rheumatoid arthritis patients treated with Etanercept.

Plasmapheresis

Plasma exchange (plasmapheresis) is particularly applied in patients with severe myasthenia, during myasthenic crisis and before or right after thymectomy [67]. During plasmapheresis, anti-AChR antibody containing plasma is separated from whole blood and replaced by albumin or plasma of healthy individuals. It mediates rapid albeit temporary improvement, which usually lasts about 4-6 weeks before patients produce again anti-AChR antibodies. Its limiting factors are difficulties with venous access, requirement of expensive equipment and side effects such as hypotension and coagulation.

Intravenous Immunoglobulin

Intravenous Immunoglobulin (IVIg) is used in autoimmune and systemic inflammatory disorders [68]. It is prepared from the plasma of several healthy donors and has high immunomodulatory effects. The mechanism of action of IVIg is complex and not very well-defined [69]. IVIg appears to be as efficient as plasmapheresis and is likewise applied in patients with severe symptoms and pre- and post-thymectomy [70]. In the last few years, IVIg has overtaken plasmapheresis as a treatment, even though it remains a rather costly therapy.

Thymectomy

Since the first successful removal of a thymic lesion in 1939, thymectomy has become part of the standard therapeutic treatment. In MG patients with thymoma, the thymus is removed to avoid spreading of the tumor [71]. In non-thymomatous MG, thymectomy is performed to reduce severity of MG and can actually alter the course of the disease, even though improvement is not immediate and can appear delayed. Within 2 to 5 years after thymectomy, about 40% of patients will be in complete remission and another 50% will be significantly improved [23].

The best response to thymectomy occurs in young, female patients with anti-AChR antibodies during the early stage of disease [72]. It remains controversial, at what time thymectomy should be performed (with respect to MG onset, the course of the disease, and patient's age), how the procedure should be done (surgical techniques), if patients with ocular MG should undergo operation and whether seronegative and MuSK MG patients benefit from thymectomy [73],[74].

1.3 EXPERIMENTAL AUTOIMMUNE MG

The search for new therapeutic approaches for MG with a higher specificity, milder side effects and a weaker global impact on the immune system requires reliable experimental animal models. Spontaneous myasthenia is observed in cats, dogs and horses including circulating anti-AChR antibodies and thymic abnormalities [75]. However, availability and manipulation of these animals is complicated. Most of the research on animals therefore relies on experimentally induced MG (EAMG) [76].

Active Induction

EAMG can be triggered by active immunization with AChR peptides, AChR subunits, recombinant fragments of muscle AChR or Torpedo AChR (T-AChR) isolated from Torpedo electrical organ, which presents a rich reservoir of this molecule [77]. The first induction of MG in animals was done by Patrick and Lindstrom in 1973, who immunized rabbits with AChR in complete Freud's adjuvant [13]. As the course of the disease was very drastic and led quickly to death of the animal, different

species had been tested to induce MG-like disease, amongst them guinea pigs, goats and primates. Nowadays, the most commonly used animals for EAMG are mice or rats [77]. EAMG is more difficult to be induced in mice than in rats and usually requires several immunizations with T-AChR, which results not always in development of MG symptoms. On the other hand, EAMG with mice allows the use of transgenic knockout mice, which permits studies that are not possible in rats.

Susceptibility to EAMG depends not only on the animal species, but also on gender, age and genetic background. Female young Lewis rats or female young C57BL/6 mice are currently most widely used as they are very susceptible to MG. In these models, EAMG occurs with a short acute phase and a chronic phase, which reflects well the course of the human disease. Further similarities between animals and humans include muscle weakness especially in the upper half of the body, decremental response in the repetitive nerve stimulation test, increased susceptibility in young females, temporary improvement after administration of AChE inhibitors, presence of serum anti-AChR antibodies, IgG and C3 complement components at the NMJ, loss of muscle AChR, MHC class II allele association and presentation of AChR epitopes [78]. However, it is important to know that while EAMG mimics well the muscular pathology of MG patients, the thymus never shows abnormalities, in contrary to the human disease [79].

The effect of novel therapeutic treatments is evaluated by the clinical score of EAMG established by Lennon *et al.* [80]. The score ranges from 0-4 and is determined according to changes in weight, posture, activity and grip. Beside clinical scores, assessment of EAMG depends on histological changes at the NMJ, AChR content on muscle cells, anti-AChR antibody titer and cytokine profiles [77].

In 2006, two animal models for MuSK-MG were established based on the immunization of rabbits with MuSK protein and by immunizing mice with the recombinant extracellular domain of MuSK, respectively [81],[82]. The MuSK-injected mice exhibited a neuromuscular junction derangement with sever fragmentation of postsynaptic AChR, denervation and muscle atrophy, which correlated with

low MuSK mRNA levels. Facial muscles were more affected than leg muscles as commonly observed in MuSK-MG patients [83].

Passive Induction

EAMG can also be induced by passive immunization with monoclonal or polyclonal anti-AChR antibodies, lymphocytes, thymocytes or serum from MG patients resulting in transient MG symptoms [84], [77]. Recent studies showed that transplantation of explants from a hyperplastic MG thymus under the kidney capsule in immunodeficient SCID mice can cause AChR loss and muscle weakness. The hyperplastic thymus contains all components to trigger MG including AChR-expressing myoid cells and autoreactive T and B cells. The anti-human AChR antibodies generated by thymic transplant crossreact with mouse AChR and mediate MG symptoms [85],[86].

While EAMG is an appropriate tool to perform preclinical studies and to assess the effect of the anti-AChR immune response, it did not answer the question, what triggers the autoimmune response. The fact that the thymus is not altered in EAMG models is an important issue. The immunization with T-AChR probably bypasses the implication of the thymus, which is most likely involved in the onset of the disease. Studies in EAMG also showed that the anti-AChR attack is not maintained by itself suggesting that MG patients must have factors that initiate and sustain the immune response. In this context, the thymus could play a major role.

1.4 NEW POTENTIAL THERAPIES

Innovative therapies derive from the need of drugs that combine efficacy and specificity. The search of new treatment for MG often relies on myasthenic animal models. Lately, several therapeutic approaches have been successfully tested in animals and are now waiting to be confirmed in humans.

- Cell-based therapy: The *ex vivo* generation of AChR-pulsed dendritic cells (DCs), the modulation of antigen-specific polyclonal regulatory T cells (Tregs) and the administration of mesenchymal stem cells (MSC) were shown to be effective in prevention and suppression of

EAMG animals. Cell specificity and cell numbers are the challenges to overcome when applying these cellular treatments to humans [87],[88],[89].

- Oral and nasal tolerance: The administration of recombinant fragments of the α -AChR subunit to rats and synthetic AChR peptides to mice reduced clinical symptoms and the autoantibody titer by induction of anti-inflammatory cytokines, notably TGF- β . Oral tolerance was successfully applied to animal models for multiple sclerosis, rheumatoid arthritis and type I diabetes [90]. However, the availability of large amounts of non-immunogenic autoantigens or recombinant AChR fragments complicates the transfer of this approach to humans [91],[92].
- Complement inhibitors: Complement activation by anti-AChR autoantibodies is part of the attack on the NMJ. Previously, experiments on EAMG animals treated with complement inhibitors showed reduced complement deposition at the NMJ as well as reduced severity of the disease. This therapeutical approach could therefore be of major interest for an application in humans [93].
- Modulation of AChE expression: Oral antisense oligonucleotides can cause inhibition of targeted gene transcription, by interaction with specific complementary mRNA. EN101 antisense (Monarsen) interferes with the transcription of the *AChE* gene and thus decreases the production of the protein. The oral or intravenous application of Monarsen to myasthenic animal had shown promising short- and long-term therapeutic effects. Monarsen is now being investigated in a phase II study on MG patients [94],[95].
- Protection of AChR at the NMJ: Reduced modulation of AChR was achieved in myasthenic animals by an increased expression of the receptor-associated protein rapsyn. Rapsyn anchors the AChR to the membrane and the cytoskeleton and thus makes it resistant to modulation by autoreactive antibodies even in the presence of activated complement. Protection of the AChR was also achieved by competing IgG4 anti-AChR antibodies that blocked the MIR without activating the complement system or cross-linking the AChR. The reduced modulation

prevented disease activity and was therefore proposed as a treatment strategy for MG patients [96],[97].

- Specific removal of anti-AChR antibodies: Recombinant extracellular domains of the different AChR subunits were coupled to carriers, which were used to immunoadsorb anti-AChR antibodies from the serum of MG patients. The procedure was sufficient to deplete pathogenic factors from MG sera. Further improvements are necessary to increase the efficiency of antibody removal in order to undergo clinical trials [98].

Importantly, the treatments that aim the modulations of the complement, the AChE or AChR expression and the removal of anti-AChR antibodies are only symptomatic and will not prevent the production of the pathogenic autoantibodies. As soon as these treatment stop, the disease will start again.

2. THYMUS

2.1 STRUCTURE AND FUNCTION

2.1.1 GENERALITIES

Until the discovery of its functions 50 years ago, the thymus has been a mysterious organ for centuries. Performing sacrificial rites, the ancient Greeks had already noted a mass of tissue in the chest above the heart of young animals. They defined this ‘mass’ as the seat of the soul and therefore termed it θυμός (thumos) meaning heart, soul, life [99]. In 1777, the anatomist William Hewson described that the thymus “*exists during the early periods of life and is filled with ‘particles’ that resembled those in blood and lymph*” [100]. In the beginning of the 19th century, the Scottish hematologist John Beard concluded from histological observations that the thymus “*must be considered the source of all the lymphoid structures of the body ... For just as the Anglo-saxon stock has made its way from its original home into all parts of the world ..., so the original leukocytes starting from the birth place in the thymus have penetrated into almost every part of the body and have*

there created new centers for growth, for increase, and for useful work for themselves and for the body” [101].

The function of the thymus as a lymphocyte-producing organ had been confirmed by the early 1950s, nevertheless immunologists refused to attribute any immunological function to it, as most of the lymphocyte were dying within the thymus [102]. Only one decade later, it became accepted that the thymus was more than a graveyard for lymphocytes and that it was actually responsible for the development of immunologically competent cells. Ever since, enormous progress has been made in understanding the development of the thymus and its role in establishing immunity and tolerance.

The thymus is uniquely found in vertebrates and is located in the pericardial mediastinum behind the sternum. It is the first lymphoid organ that appears during embryogenesis [103]. First, the thymic endothelium develops and gets organized in a loose network separated by developing vasculature; as a second event, lymphatic precursors from developing hematopoietic tissue immigrate and colonize the thymus to finish the differentiation of the thymus as a lymphoepithelial organ. The functional thymus is composed of two lobes that arise from separate primordium. The lobes are only connected by a loosely woven connective tissue, which gives rise to septae that subdivides each lobe into a number of irregular lobules. Each thymus lobule consists of a peripheral cortex, a central medulla and perivascular spaces.

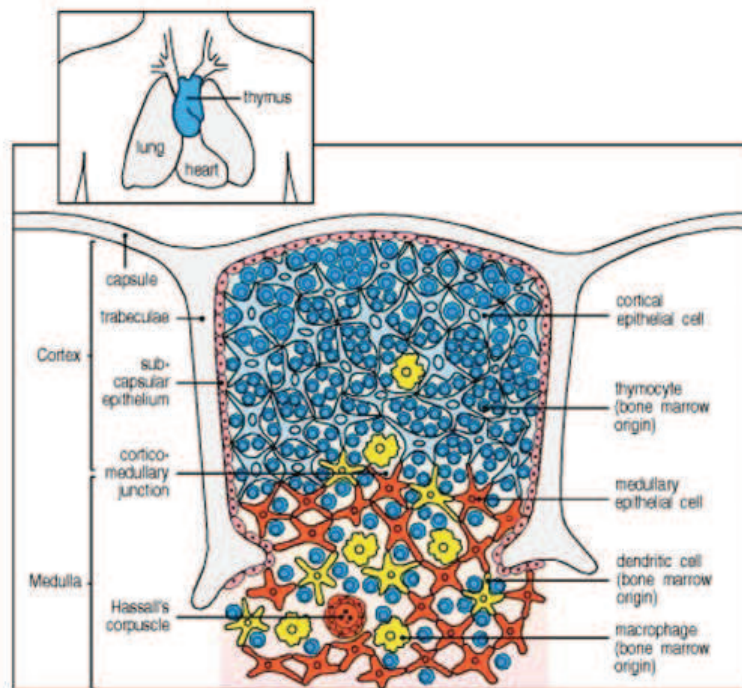


Figure 3: Cellular organization of the human thymus
(adapted from Janeway CA Jr *et al.*, Immunobiology, 2001)

The thymus is the site of T-cell maturation and selection, which is supported by the thymic microenvironment that are formed by TECs, myoid cells, fibroblasts, endothelial cells and hematopoietic cells such as dendritic cells (DCs) and macrophages (figure 3). The thymus reaches a maximum weight of 35 grams during pre-adolescent period, followed by an involution process during which it atrophies. Despite the decrease in cellular density, the adult thymus still contains thymocytes and maintains the distribution of the principal thymocyte subsets indicating that the human thymus remains active during adult life [104].

2.1.2 THYMIC CELLS

The thymus is composed of different kind of cells that can be categorized into stromal, endothelial or hemapoietic cells. The prinicpal cell types are described in the following paragraph:

Thymic epithelial cells

TECs represent the main cell type amongst thymic stromal cells and include cortical and medullary TECs (cTECs and mTECs). During the development of the thymus cTECs and mTECs derive from common progenitor cells generated from the endoderm. The differentiation of TECs depends on a panel of transcription factors including Tbx1, Hoxa3, Pax1 and Foxn1 [105]. Both mTECs and cTECs express MHC class II and the epithelial cell adhesion molecule (EpCAM), but can be phenotypically distinguished by the selective expression of keratins 8 and 18 by cTECs, and keratins 5 and 14 by mTECs. In cortical regions, cTECs are embedded between densely packed immature thymocytes and mainly mediate their positive selection. In medullary regions, mTECs are arranged more loosely and due to their promiscuous gene expression of a wide range of self-antigens, mTECs play a crucial role in the establishment of central (thymic) tolerance by depletion of autoreactive T cells (see below). Hassall's corpuscles are also observed in the medulla of the thymus. Hassall's corpuscles are rare in rodents compared to humans. They are formed by concentrically arranged epithelial cells that could correspond to highly differentiated TECs. Their precise function remains unclear, even though studies had shown that they might be involved in the removal of apoptotic thymocytes [106]. They produce molecules such as IL-7, TGF- α , CD30 ligand or SDF-1 suggesting that they may actively communicate with other thymic cells and might be implicated in the maturation of thymocytes [107], [108],[109],[110]. Lately Hassall's corpuscles were found to produce thymic stromal lymphopoietin (TSLP). It was therefore proposed that they instruct DCs and induce CD4⁺CD25⁺ Tregs [111].

Myoid cells

Myoid cells in the thymus correspond to a rare cell population localized in the medulla and at the cortico-medullary junction [112]. They resemble skeletal muscle cells, as they express muscle-specific proteins such as MyoD, desmin, troponin T, rapsyn, utrophin, and are the only cells known to express a functional AChR outside muscles [29]. The exact function of myoid cells is not clearly defined, but they are known to protect thymocytes from apoptosis and can also modulate their differentiation [113].

Endothelial cells

The microcirculatory system in the thymus consists of both blood and lymphatic vessels [114]. The blood capillaries and postcapillary venules in the thymus are characterized by a double-walled structure. They are present at the cortical-medullary junction and in perivascular spaces (PVSs), a third anatomic region next to cortex and medulla that fills with adipose tissue and lymphoid cells during age. Blood vessels in the thymus probably serve as an entry site for pro-thymocytes as well as an exit site for mature T cells [115]. The lymphatic capillary network had been detected throughout the thymus in different thymic compartments. Lymphatic vessels are frequently adjacent to blood vessels and can be found in the interlobular connective tissue, the capsule, the medulla and PVSs. Lymphatic vessels in the thymus are exclusively efferent mediating T-cell efflux from the thymus into adjacent lymph nodes [103].

Hematopoietic cells

Being the organ of T-cell selection, the thymus is mainly composed of thymocytes at different stages of differentiation as described in the next paragraph. Nevertheless, other hematopoietic cells such as DCs, macrophages and B cells can be found in the thymus, albeit they present a minor component of thymic cells.

Thymic DCs represent 0.5% of all thymic cells [116]. Two different subsets of DCs are described in the thymus: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Most thymic cDCs derive from precursors residing within thymus, but a minor subset is of extrathymic origin [117]. Localized at the cortico-medullary border and in medullary regions, thymic cDCs cross-present self-antigens to developing thymocytes and thus play a crucial role in negative selection of thymocytes as well as central tolerance induction [118].

pDCs are more abundant in the thymus than cDCs [116]. Upon activation, they are capable of producing massive amounts of IFN-I, but their role in the steady state thymus remains undefined. Recent studies suggested that pDCs might be involved in establishing immune tolerance by driving

Treg development [119]. It is, however, possible that their function simply consists in protecting the thymus from viral infection.

Macrophages can be identified in cortical and medullary regions by expression of CD68, ED1 and F4/80 cell markers [120, {Soga, 1997 #722],[121],[122],[121]. It is not completely clear from where thymic macrophages derive. They could derive from circulating monocytes, which enter the thymus through PVSs and/or they could be generated on the spot from multi-potent intrathymic cell progenitors [123], [124]. Macrophages may play a role in the removal of apoptotic thymocytes that underwent negative selection, but their exact function in the thymus remains unclear [125].

Thymic B cells represent about 2% of total thymic cells and can be found in the thymus at fetal, postnatal and adult phase with increasing number after the age of ten years [126]. They express CD19, CD20 and CD22, while they are negative for CD21 or surface IgG [127]. B cells preferentially localize around Hassall corpuscles in the medulla area and the PVS and seem to traffick between these compartments [128],[129],[126]. As medullary B cells bind closely to thymocytes, forming rosettes, they were thought to participate in T-cell selection. Recently, Akirav *et al.* have demonstrated that thymic B cells can regulate the expression of certain self-antigens by lymphotoxin- α and- β production [130].

2.1.3 T CELLS: MATURATION BY NEGATIVE AND POSITIVE SELECTION

T-cell maturation is a multi-step process occurring in the thymus. Once precursor cells colonize the thymus, they mature into functional T cells moving from the subcapsula, through the cortex into the central medulla. During this process, they receive signals for receptor gene rearrangement, for positive and negative selection and for export. Only about 1% of the cells that entered the thymus survive the selection processes and are exported to the periphery [131].

Entry of T-cell progenitors into the thymus

The colonization of the human thymus with lymphoid progenitor cells during embryogenesis begins at eight weeks of gestation via chemokine-mediated attraction to the primordium. In the postnatal

thymus, lymphoid precursor cells enter through blood vessels which are localized at the cortical-medullary junction. Their migration is primarily regulated by adhesive interaction between P-selectin on thymic endothelium and P-selectin glycoprotein ligand-1 (PSGL1) on circulating cells. The seeding of both the developing and the postnatal thymus is not continuously but occurs in waves [132].

The different stages of T-cell maturation are often characterized by the expression of CD4 and CD8. The T cell-lineage progenitors that just arrived to the thymus do not express CD4 nor CD8 and are therefore termed double negative (DN) thymocytes [133] (figure 4). The development of DN thymocytes is further subdivided in different stages depending on the expression of CD25 and CD44: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$). During the switch from DN2 to DN3, thymocytes migrate towards the subcapsular region and receive survival signals from cTECs such as delta-like ligands or IL-7. In parallel to their migration, DN thymocytes undergo developmental programs, during which they rearrange the genes encoding for the T-cell receptor (TCR). At this stage two lineages of T cells diverge expressing either $TCR\alpha\beta$ or $TCR\gamma\delta$. Successful assembly of $TCR\beta$ with pre- $TCR\alpha$ and T cell co-receptor CD3 leads to the commitment of the $TCR\alpha\beta$ lineage, representing 95% of all T cells. Next, DN3 thymocytes localized at the subcapsular region of the cortex downregulate CD25 and become DN4 thymocytes, which are the immediate precursor cells for $CD4^+CD8^+$ double positive (DP) thymocytes.

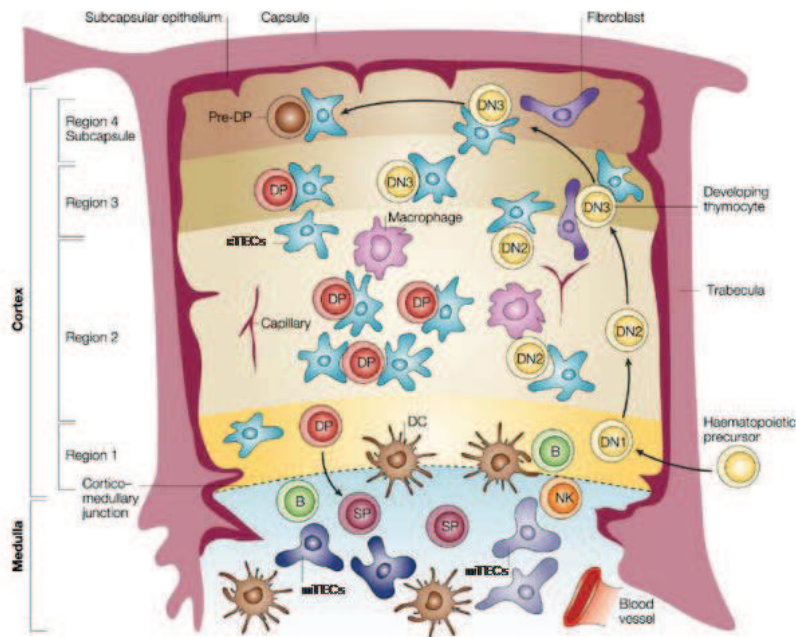


Figure 4: T cell development in the thymus
(adapted from Blackburn *et al.*, Nature Reviews Immunology, 2004 [133])

DP thymocytes in the cortex – positive selection

DP thymocytes expressing TCR $\alpha\beta$ migrate through the cortical microenvironment seeking self-antigen loaded MHC molecules presented by TECs. DP thymocytes that interact with the MHC/self-peptide complex via their TCR are selected for survival or death depending on the affinity of the interaction: thymocytes undergo apoptosis, if their interaction with this complex is too weak (death by neglect) or if they receive too strong TCR signals (clonal deletion) [134]. DP thymocytes that receive appropriate TCR signal are induced to survive and differentiate further into mature thymocytes - a process termed positive selection. Depending on the type of signals that are transmitted via the TCR, cells become either CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive (SP) thymocytes. Driven by active chemotaxis and passive flow of interstitial fluids, SP thymocytes migrate from the cortex to the medulla [132].

SP thymocytes in the medulla – negative selection

The medulla is the most important site of negative selection and thus the establishment of central tolerance. Here, SP thymocytes can interact with mTECs that express a wide range of self-antigens

from peripheral tissues such as insulin whose extrathymic expression is restricted to β -islets in the pancreas [135]. The “ectopic” expression of these tissue-specific antigens (TSAs) in mTECs is, at least partially, controlled by epigenetic factors and by a transcription activator called autoimmune regulator (AIRE) [136]. The interaction of thymocytes with TSAs expressed by mTECs allows the depletion of autoreactive thymocytes.

Not only mTECs, but also thymic DCs contribute to negative selection of developing thymocytes [137]. DCs resident in the thymus can capture and cross-present antigens including TSAs that were set free after apoptosis of mTECs. It was also described that circulating DCs can be recruited into the thymus, where they contribute to the presentation of peripheral self-antigens to induce the deletion of autoreactive thymocytes. The maturation steps of SP thymocytes are commonly identified by the expression of lymphocyte (L)-selectin (=CD62L) and CD69. Newly generated SP thymocytes are CD62^{low}CD69^{hi} cells that are not fully functional and develop into CD62L^{hi}CD69^{low} completely functional SP thymocytes, ready to be exported [138].

Exist of mature SP thymocytes from the thymus

After about 4 days in the medulla, thymocytes are exported to the periphery via blood vessels and/or lymphatic vessels in the PVS draining into an adjacent lymph nodes [139],[103],[140]. Signaling of sphingosine-1-phosphate (S1P) and chemokines seems to play a role in guiding mature thymocytes from the adult thymus to the circulation [141]. Thymocytes are known to express S1P receptor 1 and could thus be attracted by S1P in serum, where it is expressed in higher concentrations than in most tissues. The role of chemokines in T-cell exit is described below. Mature T cells were reported to recirculate back to the medulla in the thymus where they may contribute to positive selection[142],[143].

2.2 PATHOLOGICAL ROLE

2.2.1 THYMIC DISORDERS

DiGeorge syndrome

DiGeorge syndrome is a rare congenital disorder whose hallmark is a non-existent or hypoplastic (underdeveloped) thymus leading to the absence or low numbers of naive T cells and thus severe primary immunodeficiencies. In young, athymic patients, thymus transplantation is sometimes performed as a treatment [144]. DiGeorge syndrome is caused by a small deletion in the 22nd chromosome containing 20 to 30 genes. The precise function of these genes is unclear, but they seem to be critical for the normal development of several tissues. Not only the thymus but also other organs show an atypical development in DiGeorge syndrome patients including the heart, the thyroid and the kidney.

Thymic hyperplasia

Thymic hyperplasia is subdivided into two categories: True hyperplasia and lymphoid follicular hyperplasia [145]. A true hyperplastic thymus exhibits a symmetrical, diffuse enlargement that affects both cortex and medulla with preservation of the normal thymic architecture and organization. True hyperplasia can occur in association with Grave's disease, acromegaly (overproduction of growth hormones) or red-cell aplasia (anemia of the erythrocyte precursors) but also after stress, such as chemotherapy, corticosteroid therapy and irradiation. In these cases, the thymus may first undergo atrophy during the stressful events and then grow back to an even larger size than originally. This phenomenon is termed "rebound hyperplasia" and occurs preferentially in children or young adults.

Lymphoid follicular hyperplasia is characterized by a chronic inflammation and the ectopic presence of GCs [146], which is a hallmark of MG - as fully described in the paragraph 2.2.3. Follicular hyperplasia in the thymus is also observed in some case reports of patients with other autoimmune

diseases such as multiple sclerosis, rheumatoid arthritis or Grave's diseases [147]. In those cases, GC development is - unlike in MG - probably due to a secondary rather than an initial event [148].

Thymic neoplasms

Tumors of the thymic epithelium consist of thymoma or thymic carcinoma [149]. Thymomas are often further subdivided into “noninvasive/ benign” thymomas (70%), which are encapsulated tumors restricted to the thymus, and “invasive/ malignant” tumors, which grow and may spread out in adjacent structures [146]. Thymic epithelial tumors commonly appear as a mass of soft-tissue in the anterior mediastinum and can differ in size [150]. They occur equally in men and women and usually appear at the age of 50 to 60 with one third to one half of thymomas being associated with MG, as described in the following paragraph. Thymic epithelial tumors are distinct from non-epithelial primary thymic tumors such as lymphomas or germ cell tumors. A lymphomatous thymus shows an enlargement either caused by the proliferation of resident lymphatic cells or by cell infiltration of neighboring lymph nodes. Thymic germ cell tumors originate from primitive germ cells that were misplaced in the mediastinum during embryogenesis and become obvious at adolescent stage.

2.2.2 THYMOMA-ASSOCIATED MG

10-15% of MG patients display a thymoma [151]. Thymomas are heterogeneous tumors that are composed of neoplastic TECs and often a variable number of neoplastic thymocytes. From all human tumors, thymomas show the highest frequency of associated autoimmune diseases and production of autoantibodies including systemic lupus erythematosus, autoimmune hepatitis or rheumatoid arthritis [152]. About 30-50% of thymoma patients develop MG (paraneoplastic MG) [153]. Besides extremely rare exceptions, thymomatous MG patients are exclusively AChR seropositive patients, who develop additionally autoantibodies against components of striated muscles [154],[155]. Thymomatous MG can appear in all age groups but is prevalent in patients after the age of 40. The etiological background and the events that induce paraneoplastic MG remain unclear. A minority of patients could associate

the disease-onset with pregnancy, pathogenic infections or traumatic experiences, but in most cases no triggering event could be identified [156].

Despite the presence of a tumor, the thymus still delivers signals for the homing of thymocyte precursors and is functional in T cell differentiation. Intratumorous T-cell maturation occurs in almost all MG thymoma patients and leads to an export of higher numbers of CD4 T cells compared to non-thymomatous MG [157],[158]. Besides, MG-thymomas show an enrichment of autoreactive T cells against the α - and ε -subunit of the AChR [157]. The development and activation of autoreactive T cells may be related to the reduced number of Tregs in thymomas [159]. Marx *et al.* therefore proposed that non-self tolerant T cells are selected inside the thymoma during the pre-myasthenic phase of tumor growth. The naïve, autoreactive T cells may then exit the tumor site to migrate to the non-tumorous part of the thymus, where interaction with APCs and B cells mediate T cell activation. The formation of GCs can therefore sometimes be detected in areas of the thymus that are not affected by the tumor. However, autoreactive T cells could also settle in other peripheral organs such as LNs, spleens or the blood, as a complete removal of the thymus plus thymoma is often not followed by a decrease of the autoantibody titer [160].

In spite of the common symptoms, several evidence indicate that the pathogenesis of thymoma-associated MG differs from the one of MG with thymic hyperplasia:

1. Medullar areas are absent in more than 95% of paraneoplastic MG patients, while the medullar structure in patients with a hyperplastic thymus is generally preserved.
2. Globally, there is no intrathymic production of anti-AChR autoantibodies in thymomatous MG patients, which may be linked to the absence of AChR-expressing myoid cells [161].
3. Thymomatous MG does not have a gender prevalence
4. No MHC-association is known for MG patients with thymoma, but recently a protective effect of HLA-02 and/or HLA-A25 was described [162].
5. No AIRE expression was observed in the thymus of paraneoplastic MG patients [163].

6. Unlike in MG patients with hyperplastic thymus, thymomatous MG patients exhibit a thymic production of autoantibodies against IL-12, IFN- α and IFN- ω , which are a hallmark of the AIRE deficiency syndrome [164], [165].

Even though progress has been made in understanding the pathological mechanisms in paraneoplastic MG, some questions remain unanswered: Why is MG so predominant among the autoimmune disorders associated with thymomas., what is the role of AIRE deficiency and what activates the self-reactive T cells and thus initiates MG symptoms?

2.2.3 THE HYPERPLASTIC THYMUS IN MG

General thymic changes

In about 75% of MG patients the thymus shows morphological and functional abnormalities and is the site of considerable antibody production [166],[167]. 80% of male MG patients older than 50 years and female patients older than 60 years develop a thymoma [168]. In young MG patients under the age of 40, the thymus is characterized by follicular hyperplasia [169] (figure 5, left). The hyperplastic thymus is associated with high serum levels of anti-AChR antibodies, while involuted thymuses are associated with low levels [170]. Strikingly, more than 90% of patients with thymic hyperplasia are women (figure 5, right). While the epithelial architecture of the hyperplastic thymus is preserved with well-defined medullary and cortical areas, the structure is usually modified. Indeed, the hyperplastic MG thymus is not only characterized by the development of ectopic GCs but also by active neoangiogenic processes with development of high endothelial venules (HEVs) and lymphatic vessels [171]. At the cellular level, changes affect TECs, lymphocytes and antigen presenting cells (APCs), which secrete inflammatory cytokines and chemokines. These changes are accompanied with alterations of the extracellular matrix including the development of an unusual connective framework that contains collagen types I, III, and IV, as well as laminin and fibronectin. The basement membrane presents some discontinuity, which is in contrast with the continuous line pattern found in the normal thymus. Interestingly, these abnormalities are consistently found in close proximity to GCs [172].

These thymic changes affect only AChR and seronegative MG patients, while the thymus in MuSK MG patients does not display abnormalities and is most likely not involved in pathogenesis [173].

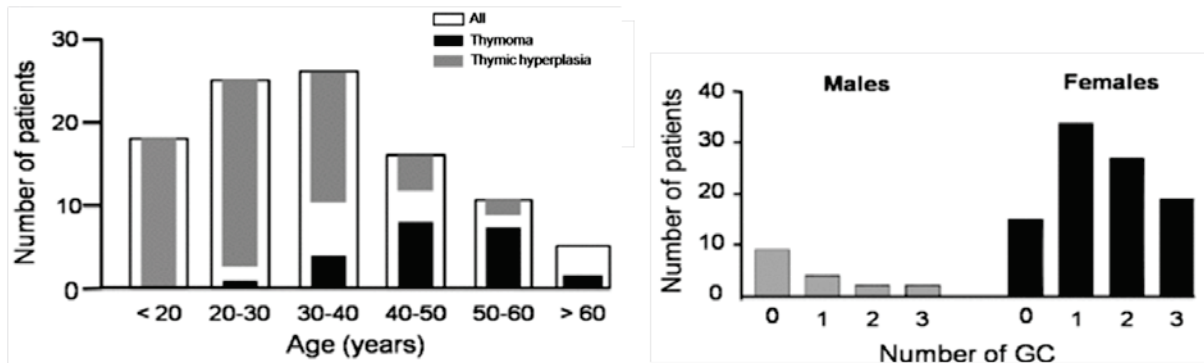


Figure 5: Thymus pathology in MG
(adapted from Le Panse *et al.*, Annals of the New York Academy of Sciences, 2004 [174])

B cell infiltrations and ectopic GC development

One of main characteristic of the MG thymus is the presence of B cells that form GCs [169] (figure 6). GCs are usually found in peripheral lymph nodes or in chronically inflamed tissues and are the site of intense B-cell proliferation, differentiation and selection. Ectopic GCs in the MG thymus contain a heterogeneous population of B cells including naïve and circulating memory B cells that undergo clonal proliferation and somatic hypermutation [175].

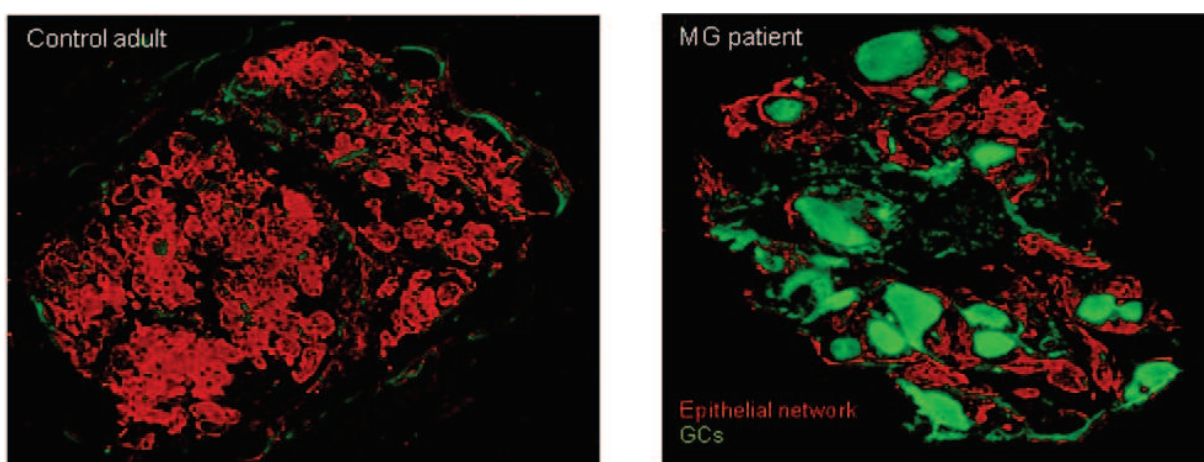


Figure 6: GC formation in the thymus of MG
Epithelial network was stained in red with antikeratin antibody; GCs in green were stained with anti-CD21 antibody
(adapted from Méraouna *et al.*, Blood, 2006 [176])

Transcriptome analysis confirmed the infiltration and polyclonality of B cells in MG thymus [177]. Purified intrathymic B cells from MG patients express activation markers such as CD23, CD25, B8.7 and were shown to spontaneously secrete IgGs and anti-AChR antibodies *in vitro* [178],[179]. The anti-AChR antibodies vary in their epitope recognition suggesting that they are produced by different clones of AChR-specific B cells [175]. Survival of B cells might be maintained by B-cell supporting factors, such as BAFF and APRIL, which are mainly produced by thymic macrophages in MG [180].

Active neoangiogenic processes

The MG thymus was recently described as a site of active angiogenesis with pathological development of lymphatic vessels and HEVs [171]. Under physiological conditions, HEVs and lymphatic vessels can be found in secondary lymphoid organs (SLOs), where they regulate homing of lymphocytes and DCs [181]. Morphologically, HEVs are distinct from normal venules due to their plump and thick endothelial cells and a thick basal lamina [182]. The exact mechanism of differentiation and proliferation of HEVs is not known, even though studies on mice showed that lymphotoxin signaling pathways might be involved [183]. Under pathological conditions, HEVs can appear in chronically inflamed tissues where they are associated with infiltration of peripheral cells and formation of lymphoid aggregates. A critical factor that regulates this cell trafficking is the interplay between chemokines and their receptors.

Altered T cells differentiation

As mentioned earlier, the anti-AChR antibodies are composed of different IgG isotypes. As isotype switch is a process depending on CD4⁺ T helper cells, an involvement of AChR-specific T helper cells in MG seems obvious. The role of T cells in MG was first elucidated by experiments in rats demonstrating that the autoimmune response in MG did not only involve anti-AChR antibodies but also autoreactive T cells [184]. Sommer *et al.* showed that anti-AChR reactive T cells are enriched in the MG thymus and can be purified [185]. Indeed, Wekerle *et al.* succeeded in isolating AChR specific T-cell lines from thymuses of MG patients [186].

Studies on the thymic T-cell repertoire showed that MG patients possess more T-cell clones with the V beta 8 and V beta 5.1 splice variant of the T cell receptor. V beta 5.1 T cells were especially found in and around GCs [187]. SCID mice engrafted with thymic explants or transferred with thymic cells lacked signs of pathogenicity, when V beta 5.1 T cells were blocked or depleted. This indicates a role of these cells in controlling pathogenic autoantibodies in MG [188]. The activation state of thymic T cells was demonstrated by an increased sensibility to IL-2 [178] and an overexpression of Fas, an apoptosis-inducing receptor and marker of activation, whose expression correlates with the AChR titer [189].

Besides CD4 effector T cells, thymic CD4⁺CD25⁺ Tregs were recently found to be implicated in MG pathogenesis and supposed to play a role in MG onset or maintenance. While the number of thymic Tregs is normal [190], they exhibited a functional defect characterized by high proliferation in response to mitogenic stimulation and impaired suppressive activity [191]. Due to their high expression of Fas, many MG Tregs could represent recirculated cells with a memory phenotype [174]. Recent results of our group suggest that the defective suppression in MG is not only related to Treg dysfunction but also to a decreased susceptibility of MG T effector cells towards suppression, as incubation of MG T effector cells with Tregs from normal thymus did not undergo suppression (unpublished).

Implication of APCs – dendritic cells and macrophages

The implication of thymic DCs in MG has never been clearly studied. However, Xia *et al.* described an abnormal number and distribution of mDCs in MG thymus [192] and Nagane *et al.* showed that mDCs localized preferentially around blood vessels and GCs [193]. In the same areas, macrophages were found in increased numbers, but except for their secretion of B-cell supporting factors such as APRIL and BAFF their exact function is not clear [180]. As for DCs, macrophages might be implicated in intrathymic sensitization by presentation of AChR captured from apoptotic TECs or myoid cells.

Changes related to thymic epithelial cells

mTECs derived from MG thymus were shown to overproduce diverse cytokines and chemokines such as TGF- β , IL-1, IL-6, CCL5 (RANTES), CXCL13 and IFN-I [176, 194-198] and might therefore intervene at different levels of T-cell differentiation leading to a modulated function:

- As mentioned above, TECs and especially mTECs are critical for the establishment of central tolerance by the expression and presentation of a wide panel of TSAs (such as AChR subunits) to thymocytes. Perturbations or modifications of this process can result in the survival of self-reactive T cells and thus in the development of autoimmunity [61].
- In a recent study of our team, the co-culture of control Tregs with TECs from MG thymus lead to a default of control Tregs, while the co-culture of MG Tregs with TECs from control thymus protected the Treg phenotype. These data suggest an impaired function of MG TECs in the formation of Tregs (unpublished).
- mTECs were also suggested to play a role in the priming of T cells and B cells by presentation of AChR. Whether AChR priming is directly performed by mTECs themselves or via cross-presentation by DCs, is not fully understood. Recently, it was demonstrated that TECs of MG patients with a hyperplastic thymus are characterized by an ectopic presence of complement receptors and regulators indicating that they are affected by a complement attack. In particular, an upregulation of C5a receptor, deposits of complement component CD3b and expression of complement regulator CD59 were detected. Leite *et al.* therefore proposed a scenario in which T helper cells are primed by AChR-subunits expressed by mTECs leading to a stimulation of B cells and an early production of antibodies against these subunits [199]. Next, these antibodies would attack adjacent, AChR-expressing myoid cells resulting in activation of APCs, inflammation and complement-mediated damage and subsequently formation of thymic GCs.

Potential role of myoid cells

In contrast to mTECs, which express low levels of AChR subunits, myoid cells express high levels of AChR subunits, which form functional AChRs [29]. These cells are the main source of AChR in the thymus. In MG patients, myoid cells are especially found inside or around GCs [200]. As they are negative for MHC II, they are most likely incapable of antigen-presentation, but it is possible that APCs such as DCs which are often located around myoid cells, uptake AChR released from apoptotic myoid cells and cross-present it to lymphocytes [201]. Myoid cells of the MG thymus are particularly sensitive towards complement, as they are expressed by a low or non-detectable level of early complement regulators such as CD55, CD46, CR1, and CD59. Myoid cells in vicinity of GCs were particularly found to be positive for the complement compounds C1q, C3b and C9 suggesting a damage by the complement system, which contributes to the formation of GC and autoantibody diversification [199].

Inflammatory signature in MG thymus

Transcriptome studies on MG thymus have demonstrated an inflammatory signature in MG thymus. While no direct overexpression of IFNs was observed [177], [202], multiple genes that are controlled by IFN-I (=IFN- α and IFN- β) or IFN-II (=IFN- γ) were upregulated [203],[174]. Le Panse *et al.* demonstrated an effect of corticosteroids on IFN-I but not in IFN-II induced genes, suggesting that corticosteroids could act on IFN-I signaling to downmodulate thymic inflammation [174]

IFN-I is secreted by multiple cell types as an antiviral defense mechanism. Viral infections are often associated with autoimmunity. In MG, some patients relate disease onset to viral infection [204],[205],[206]. Recently Cavalcante *et al.* detected traces of Epstein-Barr virus in a small number of MG patients, however the relevance of these findings is currently subject of debate [207],[208],[209]. Other signs of viral infection in MG thymus include defective natural killer cell activity [210] and the upregulation of Toll-like receptors (TLRs), which recognize pathogen-associated molecules [211]. A viral infection could either trigger a local inflammation in the thymus

and thus mediate an autoantigen sensitization (bystander effect) or activate B and T cells due to similarities between viral antigens and self-antigens (molecular mimicry).

IFN-II was demonstrated to strongly induce AChR subunit expression in TECs and myoid cells, particularly AChR- α [203]. This effect was increased when IFN-II was applied together with TNF- α . In IFN-II knockout mice, AChR levels were decreased in the thymus but not in the muscle indicating that pro-inflammatory cytokines influence the expression level of thymic AChR and could thus be implicated in the primary steps of autosensitization [203].

3. CHEMOKINES

3.1 GENERAL PROPERTIES

3.1.1 STRUCUTRE AND CLASSIFICATION

Chemokines are a group of secreted cytokines (with the exception of the membrane-bound chemokine fractalkine) that are involved in a wide range of cellular processes mainly by the regulation of cell migration [212]. They have been detected in vertebrates, invertebrates, some bacteria and viruses. Up to now, more than 50 human chemokines have been identified, which bind to one or more chemokine receptors. The interplay between chemokines and their receptors guarantees a selective recruitment of specific cell types to particular tissue or niche.

Chemokines are small molecules of 8-10 kDa with a highly conserved structure and 20-95% sequence identity. They are characterized by the presence of four cysteine residues at the N-terminus that are critical for their tertiary structure. According to the number of amino acids between the first two cysteine residues, chemokines are classified into the C, CC, CXC or CX₃C families with X representing any kind of amino acid [213]. For their nomenclature, an “L” for ligand and a number corresponding to the order of their discovery is added. For the “C family”, there are two C chemokines, XCL1 and XCL2, which carry only one out of the four conserved cysteine residues. In parallel to this systematic nomenclature, traditional abbreviations dating back to the time when the first chemokines were discovered are still used in the literature (table2).

Another more general classification is based on the site of production and the condition of stimuli, and distinguishes “inflammatory” and “homeostatic” chemokines. Inflammatory chemokines - also known as induced chemokines - attract especially leukocytes and are produced by resident or infiltrated cells of inflamed tissues such as endothelial, epithelial, and stromal cells [214]. Production of inflammatory chemokines is stimulated by pathogenic agents or pro-inflammatory cytokines such as lipopolysaccharides (LPS), IL-1 and TNF- α . Classical representatives of this group include CXCL8, CCL5, CCL4, CCL2 and CXCL10.

Homeostatic chemokines, also called lymphoid, constitutional or housekeeping chemokines, are produced in both lymphoid and non-lymphoid organs and take part in the physiological leukocyte traffic and cell organization e.g. during hematopoiesis and immune surveillance [215]. Examples for this group are CXCL13 expressed by stromal cells in GCs and CCL21 expressed on stromal cells of the T-cell area in lymph nodes. Several chemokines can be attributed both inflammatory and homeostatic properties depending on the context, in which they are produced and have to be ascribed to both subcategories, e.g. CCL22 which can have a pro-inflammatory role in inflamed lungs but also regulate physiological cellular interaction in SLOs.

CC Family		CXC Family	
CCL1	I-309	CXCL1	GRO alpha, MGSA, GRO1, NAP-3
CCL2	MCP-1, MCAF, HC11	CXCL2	GRO alpha, GRO beta, MIP-2 alpha, GRO2
CCL3	MIP-1 alpha, LD78 alpha, LD78 beta, GOS19, Pat464	CXCL3	GRO gamma, MIP-2 beta, GRO3
CCL4	MIP-1 beta, pAT744, ACT-2, G-26, HC21, H400, MAD-5, LAG-1	CXCL4	PF4
CCL5	RANTES	CXCL5	ENA-78
CCL6		CXCL6	GCP-2
CCL7	MCP-3	CXCL7	NAP-2, CTAPIII, beta-Ta, PEP
CCL8	MCP-2, HC14	CXCL8	IL-8, NAP-1, MDNCF, GCP-1
CCL9/10		CXCL9	MIG
CCL11	Eotaxin	CXCL10	IP-10
CCL12		CXCL11	I-TAC, beta-R1, H174, IP-9
CCL13	MCP-4, Ck beta 10, NCC-1	CXCL12	SDF-1, PBSF
CCL15	HCC-1, MCIF, Ck beta 1, NCC-2, CCL	CXCL13	BCA-1
CCL15	MIP-1 delta, CC-2, MIP-5, HCC-2, NCC-3	CXCL14	BRAK
CCL16	HCC-4, LEC, ILINK, NCC-4, LEC, LMC, CK beta 12	CXCL15	
CCL17	TARC, Dendrokinine	CXCL16	CXCL16, SRPSOX
CCL18	PARC, DC-CK1, AMAC-1, Ck beta 7, MIP-4, DCtactin	CX₃C Family	
CCL19	MIP-3 beta, ELC, Exodus-3, Ck beta 11	CX₃CL1	Fractalkine, ABCD-3
CCL20	MIP-3 alpha, LARC, Exodus-1	C Family	
CCL21	6Ckine, Exodus-2, SLC, TCA-4, CK beta 9	XC11	Lptn, SCM-1 alpha, ATAC
CCL22	MDC	XC12	SCM-1 beta
CCL23	MPIF, Ck beta 8, Ck beta 8-1, MIP-3, MPIF-1		
CCL24	Eotaxin-2, MPIF-2, Ck beta 6		
CCL25	TECK, Ck beta 15		
CCL26	Eotaxin-3, MIP-4 alpha, IMAC, TSC-1		
CCL27	CTACK, ILC, PESKY, ESkin, Skinkine		
CCL28	CCL28, MEC		

Table 2: Systematic and traditional names of chemokines
(adapted from Zlotnik *et al.*, Immunity, 2000, [213])

3.1.2 CHEMOKINE RECEPTOR SIGNALING

Chemokine receptors are named according to the type of chemokine(s) they bind (CXC, CC, XC, CX₃C), followed by R for receptor and a number according to the order of discovery {Murdoch, 2000 #772}. The chemokine system is redundant with promiscuous chemokine receptors binding to several chemokines (e.g. CXCR3 binds to CXCL9, CXCL10 and CXCL11) and promiscuous chemokines binding to several receptors (e.g. CCL5 binds CCR1, CCR3 and CCR5). However a few chemokine-chemokine receptor interactions are specific. Monogamous chemokines are rather homeostatic, while promiscuous chemokines are commonly associated with inflammation (figure 7). Chemokine receptors are expressed by a large variety of cells including immune cells, endothelial cells and neurons [216]. They can be constitutively expressed or induced by activation agents such as cytokines and LPS [217].

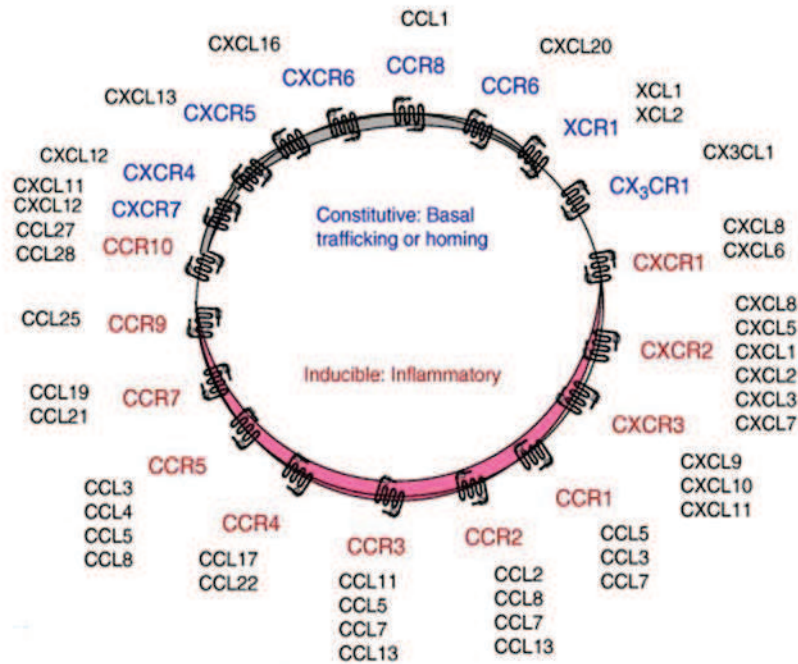


Figure 7: Chemokines and their receptors

(adapted from Johnson *et al.*, Trends in Immunology, 2005 [218])

Chemokine receptors belong to the large family of seven transmembrane domain receptors and transduce signals via G-proteins that couple to the cytosolic part of the receptor [219]. G-proteins are protein complexes composed of three distinct subunits $G\alpha$, $G\beta$ and $G\gamma$. When the molecule GDP is bound to the $G\alpha$ subunit, the G-protein complex is inactivated (figure 8, left). Once a chemokine binds to its receptor, the receptor-associated G-protein will exchange GDP against GTP and the $G\beta/G\gamma$ subunit will be set free (figure 8, right). $G\beta$ activates phospholipase C (PLC) coupled to the cell membrane. Upon activation, PLC cleaves phosphatidylinositol (4,5)-bisphosphate (PIP₂) and thus generates the two second messenger molecules inositol triphosphate (IP₃), which will trigger mobilization of Ca^{2+} , and diacylglycerol (DAG), which activates protein kinase C (PKC). This initiates signaling cascades that result in a cellular response.

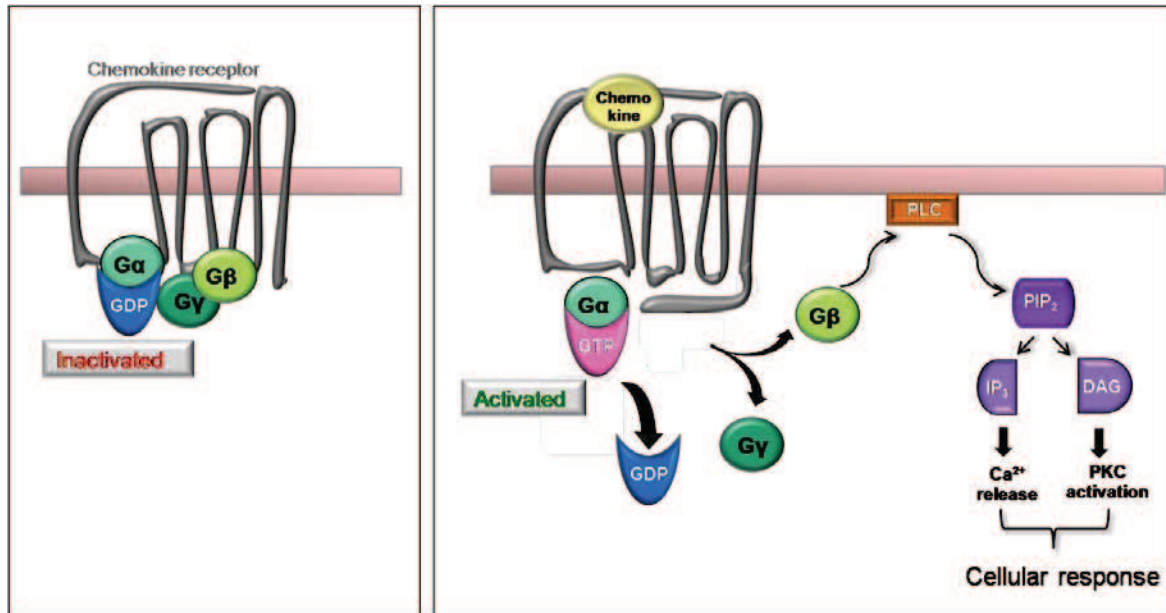


Figure 8: Chemokine signaling pathway

Left: Chemokine receptor/G-protein complex is inactivated.

Right: Chemokine binding initiates activation of the complex and triggers intracellular cell signaling

Signal transduction through chemokine-receptor interaction is fast in onset and has only a transient duration. Upon ligand binding, chemokine receptors are rapidly internalized and receptors are either degraded or recycled on the cell surface. This allows an ongoing redistribution of the receptors on the cell surface and a quick reactivity towards a chemokine gradient [214]. Apart from the functional signal-transducing chemokine receptors, members of the so-called decoy-receptor family can also bind to chemokines. Decoy receptors are structurally not able to activate G-protein dependent pathways and do not signal for cell activation and migration. They are therefore also referred to as “silent” receptors. Nevertheless, recent studies have shown that they can contribute to the establishment of chemokine gradients by degradation or concentration of their ligands [220]. The family of decoy receptors include D6, Duffy Antigen Receptor for Chemokines (DARC), CCRL2, CCX CKR, and CXCR7 [221].

3.2 PHYSIOLOGICAL ROLE OF CHEMOKINES

Due to their ability in guiding cells to target sites, chemokines are implicated in multiple physiological processes such as embryogenesis, hematopoiesis, tissue development and angiogenesis [222]. Two

processes that illustrate the working mechanism of chemokines are lymphocyte homing to SLOs and thymopoiesis.

3.2.1 LYMPHOCYTE HOMING

Leukocyte extravasation from vessels to target tissue is a multi-step cascade that includes cell tethering, rolling, adhesion, transmigration and chemotaxis. This cascade is initiated by low-affinity interactions between selectins or integrins on circulating leukocytes with their receptors such as mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), vascular cell adhesion protein1 (VCAM1) or intercellular adhesion molecule 1 (ICAM1) on endothelial cells [223]. Due to their transient binding, leukocytes will slow down and roll along the vessel wall, which allows them to come in contact with chemokines displayed on the endothelium. The interplay between chemokines and chemokine receptors activates integrins on the leukocyte surface and triggers a firm adhesion to the endothelium. As a result, the adherent cells will move across the endothelial layer and enter the underlying tissue (figure 9). Only cells that express a chemokine receptor that is capable of binding the chemokine present on the local endothelium will transmigrate to the tissue. Chemokines and their receptors thus define the selective criteria for cells entering the site of interest. Once the circulating cells are inside the tissue, they will follow a chemokine gradient that determines their precise localization.

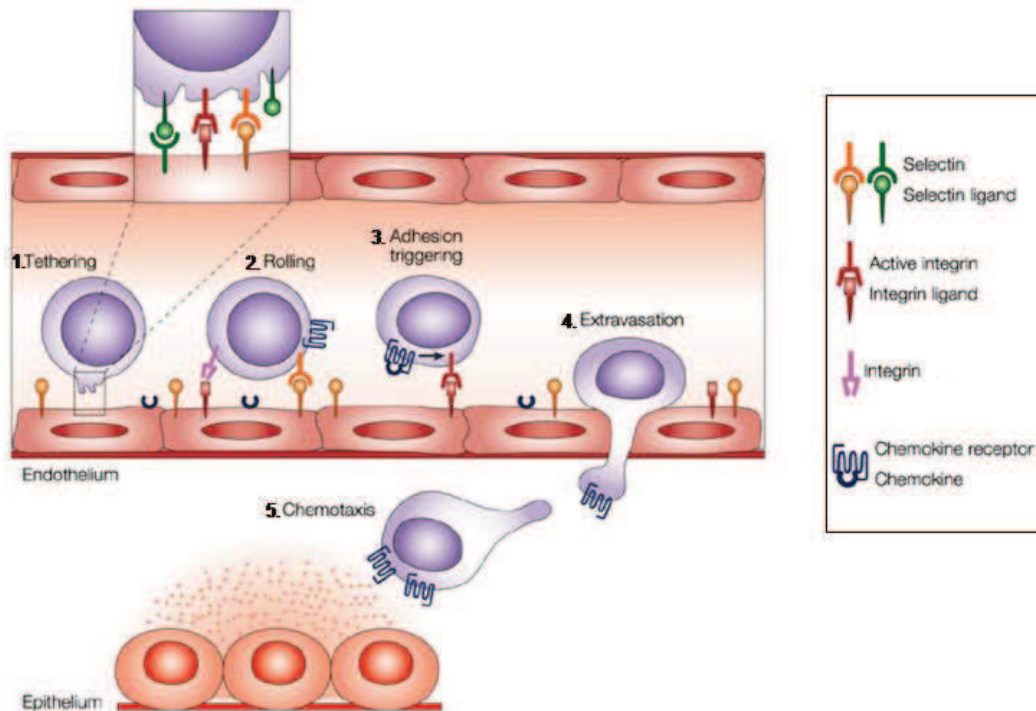


Figure 9: Leukocyte recruitment from vessels

(Eric J. Kunkel & Eugene C. Butcher, Nature Reviews Immunology, 2003, [224])

This chemokine-regulated migratory process is especially important for lymphocyte homing. Under physiological conditions, lymphocytes recirculate between the blood and the lymph by SLOs to encounter antigens on APCs. To reach lymph nodes or Peyer's Patches, lymphocytes adhere and transmigrate through HEVs. These postcapillary venules are primarily located in the T-cell zone, but also, in lower numbers, in the B-cell zone and correspond to the major entry site for lymphocytes. HEVs are distinct from normal venules by their morphology and particularly by their constitutive expression of homeostatic chemokines [225].

T-cell homing

CCL21 and CCL19 are present on HEVs in lymph nodes and Peyer's Patches and CCL21 was shown to induce binding of integrin to immobilized ICAM-1 under blood flow condition [226, 227]. CCL21 and CCL19 share the same receptor, CCR7, which is expressed by different cell types including naive T cells. Plt (paucity of lymph node T cells) mice that lack CCL21 and CCL19 show significant defective T-cell adhesion to HEVs [228]. The consequently impaired T-cell migration to lymph nodes

can be partially restored by CCL21 and CCL19 injections [229]. Mice deficient in CCR7 show a similar phenotype with a default in T-cell migration underlying the importance of this chemokine/receptor interplay for T-cell trafficking [230]. While CCL21 was found to be expressed by HEVs, CCL19 is produced by stromal cells in the areas surrounding HEVs from where it is transported to the luminal surface of HEVs [229]. This phenomenon is termed “transcytosis” and was also described for other chemokines [231],[232].

Due to its presence on HEVs, CXCL12 is another chemokine implicated in T-cell recruitment. In *in vitro* experiments, CXCL12 induced T-cell interaction with adhesion molecules ICAM1 and VCAM1 by activation of their ligands, lymphocyte function-associated antigen1 (LFA1) and very late antigen 4 (VLA4), respectively [225],[233]. CXCL12 on human umbilical vein endothelial cells mediates lymphocyte transmigration through endothelial cells [234]. Studies on rat cells showed that T-cell transmigration through cultured endothelial cells from HEV is blocked by CXCR4-antagonists [235]. The migration of CXCR4-deficient T cells to SLOs of wildtype mice, however was not impaired, indicating that CXCL12 and CXCR4 primarily have a role in T-cell extravasation rather than migration [236].

CXCL10 could also be involved in trafficking of activated T cells, as CXCL10 protein was present on HEVs [237]. Its receptor CXCR3 is not expressed on naive T cells, but can be detected on IFN-II producing CD4 T cells suggesting a role for CXCL10 in T-cell trafficking under inflammatory conditions.

B-cell homing

CXCR5, CXCR4 and CCR7 are the main chemokine receptors involved in B-cell homing to SLOs and their ligands CXCL13, CXCL12 and CCL21 can be detected on the surface of HEVs [182]. Migration of CXCR5-deficient B cells to Peyer’s Patches was significantly reduced when injected to wildtype mice [236]. In experiments using transgenic mice overexpressing CXCL13 in the pancreas, infiltration of B cell and development of B-cell zones were observed [238]. In a similar study with transgenic

expression of CCL21 in the thyroid gland, B-cell recruitment and formation of B-cell follicles was detected underlying the crucial role for these chemokines in B-cell migration [239]. In humans, CCL21 was shown to be a very potent chemokine for B cells acting more strongly on B cells than on T cells [171]. While CCL21 and CXCL13 attract naive B cells, CXCR4 seems to play a role for the migration of mature and memory B cells.

GC formation

Once naïve lymphocytes have crossed the endothelium in lymph nodes, Peyer's patches or the spleen, T and B cells need to find their respective niches: Naïve-B cells expressing high levels of CXCR5 are efficiently directed to B-cell follicles by CXCL13, and T cells migrate to T-cell areas guided by CCL21. In case of an immune response, GCs will form within the follicles. GCs consist of two distinct zones, called the dark and light zones based on their histological appearance. These histological differences are associated with functional differences: B cells in the dark zone, called centroblasts, undergo rapid proliferation and somatic hypermutation [240]. Next, the centroblasts become small, non-proliferating centrocytes. In the light zone they undergo negative or positive selection based on the affinity of their membrane bound antibody. Besides centrocytes, the light zone contains T helper cells and a network of follicular DCs (FDCs) that capture and present antigens [241]. Centrocytes that fail to bind antigen or to receive T-cell signals become apoptotic and are removed by macrophages. If the antigen recognition is successful and if centrocytes received T-cell help, they survive and exit the GC as long-lived plasma cells or memory B cells [242].

Formation of GCs is principally directed by CXCL12/CXCR4 and CXCL13/CXCR5 interaction [243]. Mice lacking CXCL13 or its receptor CXCR5 show significant defects in size and organization of GCs. CXCL13 expressed by FDCs directs CXCR5^{hi} centrocytes and GC T-cells and determines the correct position of the light zone in the GC. CXCL12 is expressed preferentially in the dark zone. It navigates CXCR4-expressing B and T cells and is required for centroblast localization in the dark zone (figure 10).

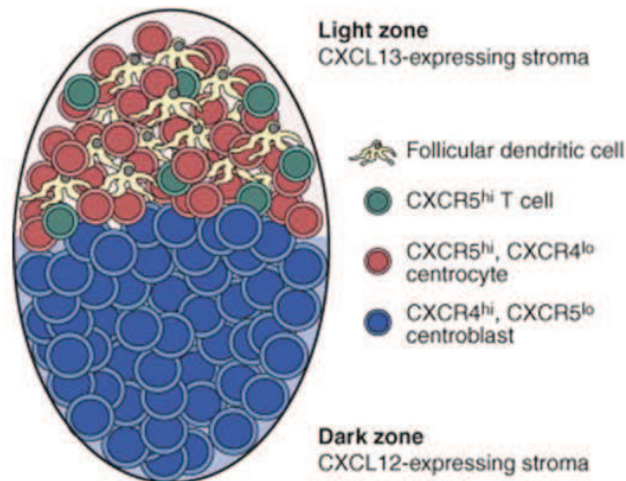


Figure 10: GC organization

(Eric J. Kunkel & Eugene C. Butcher, Nature Reviews Immunology, 2003, [224])

3.2.2 THYMOPOIESIS

Entry of pro-thymocytes, intrathymic trafficking and emigration to the periphery are events that are regulated by chemokines. Distinct thymic areas produce different combination of chemokines that direct navigation of thymocytes (figure 11). Pro-thymocytes express CXCR4 and enter the thymus via vessels localized at the cortical-medullary junction, an area rich of CXCL12 [244]. The CXCL12/CXCR4 interplay is also involved in the following differentiation steps, when DN thymocytes migrate to the subcapsular area and then to the cortical-medullary region, during which they become DP. During these developmental stages, thymocytes start expressing CCR9, which is the receptor for CCL25 expressed by TECs and DCs [245].

When thymocytes move towards the medulla, they downregulate CCR9 and become CCL25 unresponsive. In contrast to the cortical region, in which only a relatively low number of chemokines have been detected, the medulla expresses a large set of chemokines including CCL22, CCL17, CCL19, CCL21, CCL11 and CXCL16. In parallel, thymocytes that differentiate into SP T cells will upregulate CCR4 and CCR7, the receptors for CCL22/CCL17 and CCL19/CCL21, respectively [246]. Especially CCL22 and CCL17 are considered as chemokines that recruit and retain thymocytes in the medulla. The interplay between CCR7 and CCL21/CCL19 seems to be important for organizing cells

within the medulla and to guide them to vessels at the cortical-medullary region for their export out of the thymus [247].

CCL19, together with CXCL12, seems to play a crucial role in releasing mature thymocytes into the periphery [248],[249]. At high concentration, these two ligands act as chemorepellent signals, to which immature thymocytes are insensitive. The active movement away from a chemotactic stimulus (fugetaxis) is probably responsible for the emigration of mature T cells from the thymus. Even though the role of chemokines in thymopoiesis is relatively well defined, it is not completely clear, why thymocytes lacking CCL19/CCL21 or precursor cells deficient in CCR4, CXCR4 or CCR7 show no obvious defect in T-cell development.

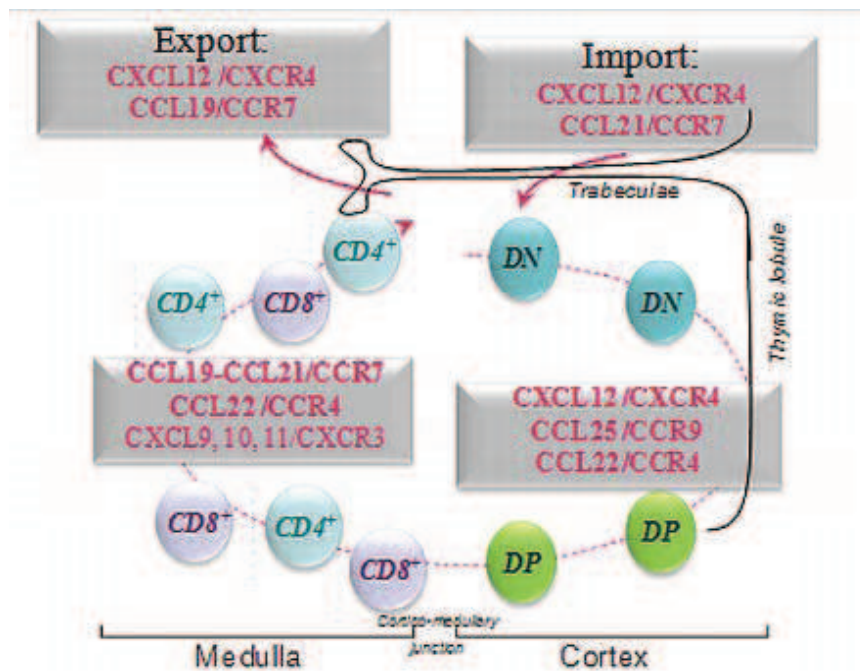


Figure 11: Chemokines in thymopoiesis
(Le Panse *et al.*, Autoimmunity, 2010, [250])

3.3 PATHOLOGICAL ROLE OF CHEMOKINES

The ubiquitous expression of chemokines requires a precise and accurate working mechanism. Disturbances and modifications of the chemokine network can have dramatic consequences, as proven by implication of chemokines in multiple disorders such as asthma, allergies and cancers [251]. As

chemokines are especially involved in the regulation of immune cell migration, they particularly contribute to autoimmune diseases [252].

Altered expression of chemokines and their receptors has been described in animal models of lupus erythematosus, diabetes and rheumatoid arthritis (RA) [253],[254],[255]. In mouse and rat models for multiple sclerosis (MS), mRNA levels of diverse chemokines (CCL1, CCL2, CCL3, CCL4, CXCL10 and CXCL12) are shown to correlate with disease progression [256]. Consistent with data obtained from animal models, clinical studies on humans demonstrated a dysregulation of chemokines and chemokine receptors in the course of certain autoimmune diseases often associated with leukocyte infiltration into chronically inflamed tissues. For example, in salivary glands of patients with Sjogren's syndrome, CXCL13 and CCL21 expression are associated with the formation of ectopic lymphoid follicles [257]. In RA and systemic lupus erythematosus, CXCL9 and CXCL10, respectively, have been proposed as markers for disease activity, as chemokine levels correlated with disease severity [258],[259].

3.3.2 CHEMOKINES IN MG THYMUS

Data from transcriptome studies combined with chemotactic assays on thymic extracts showed an increased expression of chemokines in the thymus of MG patients and especially of those with a high hyperplasia. Meanwhilen, further studies on MG thymus, EAMG models, TEC cultures and peripheral blood cells have confirmed the dysregulation of several chemokines in MG.

CXCL13 and CXCR5

Lately, our group has shown that CXCL13 is overexpressed in the thymus of all subtypes of MG patients including AChR MG patients with a low or high degree of hyperplasia and seronegative patients [176]. The overproduction of CXCL13 in the thymus was attributed to TECs and was normalized in patients under corticosteroid treatment. *In vitro* studies have shown that thymic extracts from MG patients had a strong chemoattractive effect on B cells. This effect was reduced when using anti-CXCL13 antibodies or thymic extracts from corticosteroid-treated patients. These data suggest

that the excess of CXCL13 in the thymus could be responsible for the B-cell infiltration and GC formation in MG thymus. In parallel, CXCL13 levels were found to be elevated in MG serum and were decreased after corticoid-treatment or thymectomy correlating with clinical improvement [260]. At the same time, the number of follicular helper T cells, expressing CXCL13 receptor CXCR5, was increased in MG blood compared to controls and correlated with disease activity [261]. After thymectomy or corticotherapy, their numbers decreased gradually to control levels. Saito *et al.* suggested therefore to use the number of CXCR5⁺ CD4⁺ T cells in MG serum as an index for disease severity.

CCL21/CCL19 and CCR7

Next to its role in thymopoiesis, CCL21 is associated with the formation of GCs. It is therefore not surprising, that MG thymus with a high degree of hyperplastic is characterized by an overexpression of CCL21 [177]. Berrih-Aknin *et al.* had shown by laser-capture microdissection and real-time PCR, that CCL21 was located on lymphatic endothelial vessels [171] and that it could participate to the chemoattractive effect of a hyperplastic thymus extract on T and B cells observed in chemotactic assays [177]. CCL21 was also observed around extralobular blood vessels where CD44^{high} populations accumulated [193]. It was proposed that CD44⁺ DCs may migrate to the hyperplastic thymus via the vascular system by a CCL21-dependent mechanism. These DCs were suggested to promote the priming of autoreactive-T cells by presenting self-antigens, such as AChR.

Next to CCL21, albeit less striking than CCL21, CCL19 was augmented in hyperplastic thymus [171]. No deregulation of CCL21/CCL19 receptor CCR7 or CCR11, a CCL21 decoy receptor, were observed in the periphery.

CXCL10 and CXCR3

The chemokine CXCL10 regulates cell activation and migration by binding to its receptor CXCR3 on activated T cells, eosinophils, monocytes and natural killer cells. CXCL10 levels are often increased in autoimmunity and lead to cell recruitment to inflammatory sites [262].

In EAMG CXCL10, CXCR3 and cytokines that induce CXCL10 were overexpressed by lymph node cells and in the muscle of EAMG rats [263]. Levels were reduced after EAMG suppression by mucosal tolerance induction with an AChR fragment. The relevance of these results was confirmed by similar observations in MG patients who exhibited a significant increase in CXCL10 and CXCR3 mRNA levels in muscles and also in the thymus. In MG blood, CXCR3 was specifically increased on CD4⁺ T cells suggesting an enhanced recruitment of these cells from the periphery [264]. To test the possibility of using CXCL10 signaling as therapeutic target, Feferman *et al.* used CXCR3 antagonist and antibodies blocking specifically the binding of CXCL10 to CXCR3 [265]. Both treatments were effective in the suppression of ongoing EAMG and reduction of the AChR-specific immune response.

CCL5 and CCR1/CCR3/CCR5

CCL5 is a chemokine acting on T cells, eosinophils and basophils via the interaction with its receptors CCR1, CCR3 and CCR5. As CCL5 was augmented in the hyperplastic thymus of MG patients, it might be implicated in the pathogenesis of the disease. Colombara *et al.* had demonstrated that CCL5 expression in MG TECS was increased and that CCL5 modulated migration of peripheral T cells. Its receptor CCR1 was significantly increased on CD4 and CD8 T cells and reduced after therapy suggesting an involvement of the CCL5/CCR1 signaling pathway in thymic pathology of MG [264].

CCL2 and CCR2/CCR4

CCL2 is expressed at sites of tissue injury and recruits monocytes, DCs, natural killer cells and T cells [266]. CCL2 seems to be involved in the pathogenesis of EAMG, as it is overexpressed in lymphoid organs and muscles of EAMG mice and rats [267],[268]. In SLOs, CCL2 affects the development of autoreactive Th17 cells and could likewise be implicated in EAMG [267]. While the involvement of CCL2 in EAMG seems to be convincing, no study exist so far describing CCL2 modifications in MG patients.

II. AIM OF THE STUDY

While MG is a neuromuscular disease characterized by muscle weakness, the thymus often shows morphological and functional abnormalities, such as thymic hyperplasia. The hyperplastic MG thymus contains anti-AChR autoreactive T cells and GCs with B cells producing anti-AChR antibodies. It is the primary production site of pathogenic autoantibodies and the degree of thymic hyperplasia correlates directly with the antibody-titer. The thymus is thus clearly involved in the development of MG and thymectomy is often advised to MG patients improving slowly but efficiently their symptoms (Part 4 of the PhD – review: LePanse *et al.* [250, 269][251] - published). Although progress has been made in developing therapies for MG, this disease is still incapacitating and treatments are not satisfactory. The aim of our research group is therefore to gain deeper insights into the pathophysiological mechanisms leading to thymic hyperplasia.

To this end, our team has performed a transcriptome study comparing thymic gene expression between control adults and MG patients. The results demonstrated a large number of dysregulated genes in MG confirming the inflammatory state of the MG thymus and an important B-cell infiltration, especially in hyperplastic thymus. In parallel, the expression of several chemokines was shown to be modified in MG thymus, most strikingly those of CXCL13 and CCL21. CCL21 was especially dysregulated in patients with thymic hyperplasia, while CXCL13 levels were elevated in all MG patients, whatever the pathological state of their thymus. Further studies showed that CXCL13 was overexpressed by TECs, while CCL21 was upregulated in lymphatic endothelial vessels. In the course of these investigations, active angiogenic processes with lymphoangiogenesis but also the important development of ectopic HEVs in the MG thymus were revealed. **The objective of my PhD** was therefore to identify the contribution of chemokines and HEVs to the development of thymic hyperplasia.

In SLOs and chronically inflamed tissues HEVs serve as an entry gate for circulating cells. We therefore wondered, whether thymic HEVs were capable to mobilize peripheral cells to the MG

thymus and thus contribute to disease development. To this purpose we analyzed the localization of HEVs in MG thymus and investigated if there was a link between the degree of thymic hyperplasia and the number of HEVs. To determine whether HEVs were efficient in cell recruitment, we examined peripheral cells in the environment of HEVs. In SLOs, HEVs-mediated cell migration is dependent on the chemokines displayed on the surface of HEVs. In order to identify the working mechanism of HEVs in MG thymus, we investigated their chemokine expression pattern. The analyzed chemokines were chosen by two criteria: a reported expression on HEVs in SLOs or other inflamed tissues, and a reported dysregulation in MG thymus. As we uniquely found the presence of SDF-1 on thymic HEVs, we investigated in detail its role in peripheral cell recruitment to the MG thymus and the expression of its receptor CXCR4 on thymic and peripheral cells (Part 1 of the PhD - Weiss *et al.* - Submitted).

Being a powerful B-cell attractant, CXCL13 could play a major role in B-cell infiltration and GC organization in the MG thymus. Previous studies had shown that the ectopic expression of CXCL13 in pancreatic and intestinal cells caused B-cell recruitment, formation of HEVs and upregulation of CCL21. As our team had demonstrated that the chemoattractive effect of MG thymus extracts could be inhibited by blocking CXCL13, we wanted to test if thymic CXCL13 overexpression in mice could induce B-cell mobilization and eventually lead to hyperplasia. Mice displaying a thymic hyperplasia could serve as a basis for a new MG animal model which would reflect the human diseases much better than the current MG model. Presently, MG in animals is induced by injection of the purified AChR, which leads to muscle pathology, but – in contrary to the situation in MG patients - not to thymic abnormalities. We therefore chose to generate a new transgenic mouse line with a CXCL13 overexpression in TECs. To this purpose, we created a gene construct containing the murine CXCL13 gene under the control of the Keratin5 promoter, which was microinjected into fertilized oocytes from C57BL/6 mice. We showed that these transgenic mice indeed overexpress CXCL13 in the thymus and we are currently analyzing in detail the phenotype of these mice with focus on thymic changes and their susceptibility to EAMG (Part 2 of the PhD - Weiss *et al.* - in preparation).

Next to an overexpression of chemokines and B-cell markers, the transcriptome studies of the MG thymus had demonstrated an upregulation of IFN-I-regulated genes. In addition, it had shown that the expression of these genes was normalized in patients under corticotherapy, a treatment known to reduce thymic hyperplasia. To determine whether IFN-I could be involved in thymic hyperplasia, we investigated in detail the role of IFN-I, *in vitro* and *in vivo*, on thymic changes related to MG, such as chemokine expression. Moreover, we also examined the factors that could trigger the production of IFN-I in the thymus. We especially demonstrated a specific role of PolyI:C, a dsRNA analogue, which stimulated the release of IFN-I, upregulated thymic chemokine expression and induced the expression of the α -subunit of the AChR. We also examined whether the thymic changes associated with dsRNA signaling could lead to a breakdown of AChR- tolerance (Part 3 of the PhD - Cufi *et al.* - in revision)

The results of these studies are fully described in the next chapter of the manuscript. These results help to better understand the events that induce and/or maintain thymic hyperplasia and to identify factors that could present new therapeutic targets. These treatments could involve molecules blocking the effects of chemokines and/or controlling the neoangiogenic processes in order to stop ectopic GC formation and thymic hyperplasia development in MG.

III. RESULTS

ARTICLE 1**SDF-1/CXCL12 recruits peripheral blood cells to the thymus of autoimmune Myasthenia Gravis patients****OBJECTIVE**

More than 80% of early onset myasthenia gravis (MG) patients with anti-acetylcholine receptor antibodies are characterized by thymic hyperplasia. Their thymus shows signs of B-cell infiltration, germinal center development and neoangiogenic processes. Recently, the presence of ectopic high endothelial venules (HEVs) was described in the hyperplastic thymus of MG patients. As HEVs are typically observed in secondary lymphoid organs and chronically inflamed tissues, where they mediate transmigration of lymphocytes and dendritic cells, we wondered about their role in the MG thymus and their contribution to thymic hyperplasia. We therefore analyzed their localization in the MG thymus and examined if there was a correlation between the presence of HEVs and the degree of thymic hyperplasia. We also investigated in detail the nature of cells entering the thymus through HEVs and the chemokines displayed on the surface of HEVs.

As we uniquely found the presence of SDF-1 on thymic HEVs, we investigated its role in peripheral cell recruitment to the thymus of MG patients and the expression of its receptor CXCR4 on thymic and peripheral cells.

SDF-1/CXCL12 recruits peripheral blood cells to the thymus of autoimmune Myasthenia Gravis patients [†]

Running title: SDF-1 recruits peripheral cells to the MG thymus

Julia Miriam Weiss¹, Perrine Cufi^{1*}, Jacky Bismuth^{1*}, Bruno Eymard², Elie Fadel³, Sonia Berrih-Aknin¹ and Rozen Le Panse¹

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¹ Unité mixte de recherche - CNRS UMR7215/INSERM U974/UPMC UM76/AIM - Thérapie des maladies du muscle strié, Institut de Myologie, 75651 Paris Cedex 13, France.

² Unité clinique de pathologie neuromusculaire, Institut de Myologie, 75651 Paris Cedex 13, France

³ Service de chirurgie thoracique et vasculaire et de transplantation cardio-pulmonaire, Hôpital Marie Lannelongue, 92350 Le Plessis Robinson, France.

* These authors contributed equally to this work.

Correspondence: Rozen Le Panse
Unité Mixte de Recherche
CNRS UMR7215/INSERM U974/UPMC UM76/AIM
Thérapie des maladies du muscle strié
Groupe hospitalier Pitié-Salpêtrière
105, boulevard de l'Hôpital
75651 PARIS Cedex 13 - FRANCE
Tel 0033 (0)1 40 77 81 27 Fax 0033 (0)1 40 77 81 29
rozen.lepanse@upmc.fr

Abbreviations:	AChR:	acetylcholine receptor
	DC:	dendritic cell
	GC:	germinal center
	HEV:	high endothelial venules
	mDC:	myeloid dendritic cell
	MG:	myasthenia gravis
	pDC:	plasmacytoid dendritic cell
	PNAd:	peripheral-node addressin
	TEC:	thymic epithelial cell
	SLO:	secondary lymphoid organ

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Abstract

Myasthenia gravis (MG) is a neuromuscular disease mediated by autoantibodies against the anti-acetylcholine receptor (AChR). MG is associated with thymic hyperplasia characterized by ectopic germinal centers that contain autoreactive T and B cells producing anti-AChR antibodies. The thymus is also a site of active neoangiogenic processes including high endothelial venules (HEVs). The aim of this study was to analyze the role of thymic HEVs and associated chemokines in peripheral cell recruitment and their implication in MG pathogenesis.

We observed that the number of HEVs positively correlated with the degree of hyperplasia and was normalized in patients under corticosteroid treatment. Testing various chemokines, we demonstrated that thymic HEVs selectively expressed SDF-1 mRNA and presented SDF-1 protein on the lumen side. Moreover, APCs such as monocytes/macrophages, dendritic cells (DCs) and B cells expressing SDF-1 receptor CXCR4 were detected inside and around thymic HEVs. In periphery, we showed that CXCR4 expression was reduced on peripheral myeloid DCs. In parallel, the number of mDCs and monocytes was decreased suggesting the recruitment of these cells from the periphery to MG thymus. Moreover, in corticosteroid-treated patients, all these peripheral changes were normalized; an effect that might be correlated with the decreased number of thymic HEVs by corticosteroids.

Altogether, our results suggest that HEV development and engagement of SDF-1 contribute to MG pathology by recruitment of peripheral B cells and APCs to the MG thymus.

Keywords: *Myasthenia Gravis, Thymus, Chemokines, SDF-1, High endothelial venules*

1. Introduction

Autoimmune Myasthenia Gravis (MG) is a neuromuscular disorder characterized by muscle weakness which is caused by a defective transmission of nerve impulses to muscles due to auto-antibodies against components of the neuromuscular junction. Antibodies against the acetylcholine receptor (AChR) are detectable in 85% of patients, so-called AChR⁺ MG patients (1) and a minority of patients have antibodies directed to another muscle endplate protein, MuSK (Muscle specific kinase). While the muscle is the target organ, the thymus is very often involved in the pathogenesis of AChR⁺ MG patients (2). In these patients, the thymus exhibits morphological and functional abnormalities, usually characterized either by a thymoma (15%) or by hyperplasia (55%). The hyperplastic thymus shows ectopic germinal center (GC) development and contains anti-AChR antibody producing B cells and anti-AChR activated T cells (2-5). Berrih-Aknin *et al.* have recently described the MG thymus as a site of active angiogenesis and reported on the pathological development of lymphatic vessels and high endothelial venules (HEVs) (6, 7). Under physiological conditions, HEVs and lymphatic vessels can be found in secondary lymphoid organs (SLOs), where they regulate homing of lymphocytes and dendritic cells (DCs) (8). Morphologically, HEVs are distinct from normal venules due to their plump and thick endothelial cells and a thick basal lamina (9). The exact mechanism of differentiation and proliferation of HEVs is not known, even though studies on mice showed that lymphotoxin signaling pathways might be involved (10). Under pathological conditions, HEVs can appear in chronically inflamed tissues where they are associated with infiltration of peripheral cells and lymphoid aggregates.

Efficient cell recruitment via HEVs is a multistep process initiated by the interaction between selectins on circulating cells and peripheral-node addressins (PNAds) on HEVs. Next, chemokine receptors on circulating cells bind to chemokines displayed on the lumen side of HEVs. These interactions mediate firm cell adhesion and activate integrins which bind to

adhesion molecules on HEVs and trigger subsequent cell transmigration across HEVs (9). The commitment of chemokines is indispensable in this process and several of them such as CXCL10, SDF-1, CXCL13, CCL19 or CCL21 have been described to be associated with HEVs (9). SDF-1, also known as CXCL12, has been particularly well studied. First identified as a pre-B-cell growth factor (11), it was later shown to be involved in both physiological and pathological processes e.g. in chemotaxis, survival, proliferation and activation (12). SDF-1 operates primarily by binding to its receptor CXCR4 expressed on various cell types, such as B and T lymphocytes, monocytes, dendritic cells (DCs) and progenitor cells. Recently, an additional SDF-1 receptor, CXCR7, has been identified, but its involvement in SDF-1-mediated chemotaxis is disputed (13),(14). SDF-1 has been associated with several diseases (12), including a variety of autoimmune disorders (15-17), by regulating cell infiltrations and B-cell survival. Its implication in MG, however, has so far not been investigated.

The aim of this study was to decipher the involvement of thymic HEVs in MG pathology by analyzing in detail chemokines displayed on their surface, which could favor peripheral cell recruitment leading to thymic hyperplasia. We demonstrated, for the first time, that the chemokine SDF-1 and its receptor CXCR4 play a central role in MG in thymic hyperplasia development by guiding peripheral B cells and APCs via HEVs into the thymus.

2. Materials and methods

2.1. Samples

Thymic biopsies were obtained from MG patients after thymectomy or from adults undergoing cardiovascular surgery at the Marie Lannelongue Chirurgical Center (Le Plessis Robinson, France). Thymuses were classified as follows: non-MG adults (Ad), MG patients with low thymic hyperplasia (ML; with 2 or fewer GCs per section) or high thymic hyperplasia (MH; with 3 or more GCs per section), or corticosteroid-treated MG patients (Cortico). Blood samples were obtained from MG patients and age-matched healthy donors. Only AChR⁺ MG patients without thymoma or other known diseases were included. MG patients included in this study are detailed in table S1a. All studies on thymuses and blood samples were approved by the local Ethics Committee (CCP, Ile de France-Paris 7, France - agreement N°C09-36) and were carried out in accordance to the World Medical Association's Declaration of Helsinki.

2.2. Cell isolation

To obtain thymic epithelial cells (TECs), thymic explants were cultured as described (18) and detached with a trypsin treatment after 7 days of culture. PBMCs were isolated from fresh blood using a Ficoll gradient (Eurobio, les-Ulis, France). TECs and PBMCs were stored in TRIzol (Invitrogen, Cergy Pontoise, France) at -80°C until RNA isolation.

2.3. Immunohistochemistry on thymic sections

Frozen thymic sections (7µm) were fixed in ice-cold acetone for 20 minutes. Immunofluorescent staining was performed by one hour incubation at room temperature with antibodies listed in table S1b.

Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope using 40X and 63X magnifications.

2.4. Flow Cytometry

Cell populations were identified according to their characteristic FSC/SSC profile and by staining with the following antibodies: B cells with an anti-CD19 antibody, T-cell subsets with anti-CD4 or anti-CD8 antibodies and monocytes with an anti-CD14 antibody. Myeloid DCs (mDCs) were defined as CD14⁻CD11c⁺ cells and plasmacytoid DCs (pDCs) as CD14⁻CD123⁺ cells. For the analysis of intracellular CXCR4, CXCR7 and SDF-1, PBMCs were stained for surface CD11c and CD14 antigens to identify mDCs, fixed and permeabilized with an eBioscience permeabilization Kit (Montrouge, France) and labeled with anti-CXCR4, anti-CXCR7 or anti-SDF-1 antibodies. All antibodies are listed in table S2.

Staining conditions for all antibodies were 30 min at 4°C in the dark. Cells were then fixed and acquired using BD FACScalibur. Data was analyzed with FlowJo analysis software (Treestar, San Carlos, CA). For cell sorting, PBMCs were stained with anti-CD14, anti-CD11c, and anti-CD3 antibodies to identify mDCs, monocytes and T cells, and run on a 4-laser FACS Aria high-speed cell sorter using FACS Diva acquisition software (both from BD Biosciences).

2.5. Laser-capture microdissection

Microdissection was performed as previously described (19). To label HEVs, sections were consecutively stained with anti-PNAd carbohydrate epitopes, biotinylated mouse anti-rat and streptavidin-horseradish peroxidase. Antigen was visualized with the DAB⁺ system (K3467, DAKO) and sections were counterstained with hematoxylin. HEV and medullary zones were separately microdissected before RNA extraction.

2.6. Reverse transcription and real-time PCR

Total RNA was extracted as previously described (20). 1µg of RNA was reverse transcribed for 1 hour at 42°C using AMV (Eurobio, Courtaboeuf, France) with oligo-dT (Invitrogen). RNA from laser-capture microdissection was extracted with RNeasy Micro Kit (74004, Qiagen, Courtaboeuf) and reverse-transcribed using SuperScript II with oligo-dT and random

primers (Invitrogen). Real-time PCR reactions were performed on the LightCycler apparatus as previously described (20). The primer sequences (Eurogentec, Angers, France) were as follows: (1) for SDF-1 α (F:5'- CACTGTGGCTAACATTG-3', R:5'- AGGTCCTGGTGGTATT-3'), (2) for SDF-1 β (F:5'-TAGTCAAGTGCGTCCACGAG-3', R:5'- ACACACAGCCAGTCAACGAG-3'), (3) for SDF-1 α/β (F:5'- CTTTAGCTTCGGGTCAATGC-3', R:5'-CAGCCTGAGCTACAGATGC-3'), (4) for 28S (F:5'-CGGGTAAACGGCGGGAGTAA-3', R:5'-GGTAGGGACAGTGGGAATCT-3'), (5) for GAPDH (F:5'-GATGATGTTCTGGAGAGCCC-3', R:5'- GCTGAGTACGTCGTGGAGTC-3'). All samples were normalized to GAPDH or to 28S for RNA extracted from cell cultures or tissues, respectively.

2.7. Statistical analyses

In bar graphs, results are expressed as means of different experiments or duplicate values. Error bars represent SEM. For 2-by-2 comparisons, non-parametric Mann-Whitney test was applied as specified in figure legends.

3. Results

3.1. Ectopic HEVs in MG thymus

The hyperplastic thymus of MG patients is characterized by neoangiogenesis and in particular by an abnormal development of HEVs in association with GCs (6, 7). We localized HEVs by immunofluorescent co-labeling of MG-thymus sections with anti-PNAd antibody together with antibodies against keratin for epithelial cells, CD21 for GCs or CD3 for mature T cells. In agreement with previous reports (6, 21), we show that HEVs developed around GCs and in perivascular spaces (Fig. 1A). However, we also observed that thymic HEV development occurred in medullary regions embedded within the thymic epithelial cell network in close contact with mature thymocytes (Fig. 1A).

By immunofluorescent staining, we next compared the development of thymic HEVs between non-MG adults and MG patients with low or high thymic hyperplasia and with those undergoing cortico-therapy. While HEVs were not a common feature in non-MG thymus and were detected only in low quantities in only a few donors (Figure 1B), the MG thymus displayed numerous HEVs with a positive correlation between the degree of thymic hyperplasia and the number of HEVs (Figure 1C). In addition, we demonstrated that corticosteroid treatment tends to normalize the number of HEVs (Figure 1C), which correlates with the decreased number of GCs observed in the thymus of corticosteroid-treated MG patients (19, 22). As corticoids are known to inhibit angiogenesis, it is likely that their application in MG inhibits development of thymic HEVs, which reduces peripheral cell entrance and consequently the number of GCs.

3.2. Recruitment of peripheral cells via HEVs

In secondary lymphoid organs (SLOs) and inflamed tissues, HEVs serve as an entry site for cells from the periphery (9). To determine if HEVs in MG thymus were also efficient in recruiting cells, we examined peripheral cells in the environment of HEVs. Thymic sections

were first co-labeled with anti-PNAd antibody for HEVs together with anti-CD20 antibody for B cells. Immunofluorescent staining showed that most B cells were organized in lymphoid follicles (GCs), which were encircled by HEVs. Higher magnification demonstrated that B cells were found in close association with HEVs suggesting that they could enter through HEVs into the MG thymus (Fig. 2A-B). We next analyzed the environment of HEVs for APCs. Staining of thymic MG sections with anti-CD14 antibody showed that monocytes/macrophages were scattered all over the thymus (Fig. 2C), as previously described (23), but were especially located in the lumen of HEVs (Fig. 2D). Using an anti-DC-LAMP antibody, we localized mature mDCs in the surrounding of HEVs as well as in direct contact with HEVs (Fig. 2E, F). pDCs, identified by anti-CD123 staining, were also found in close proximity to HEVs (Fig. 2G, H). These observations suggest that thymic HEVs in MG patients are functional in recruiting B cells and APCs and could actively contribute to thymic hyperplasia.

3.3. Analysis of chemokine expression on thymic HEVs in MG

In SLOs and inflamed tissues, HEVs display chemokines on their surface which mediate adherence and transmigration of peripheral cells to the target tissue (9). We therefore analyzed the chemokine expression pattern on thymic HEVs in MG. Sections of hyperplastic MG thymus were co-labeled with an anti-PNAd antibody for HEVs and antibodies against selected chemokines: CXCL9, CXCL10, CXCL11, SDF-1, CXCL13, CCL19, CCL21 and RANTES. The analyzed chemokines were chosen by two criteria: a reported expression on HEVs in SLOs or other inflamed tissues, and a reported dysregulation in MG thymus, as detailed in table S2. From all these chemokines, we exclusively observed SDF-1 α/β expression on the lumen side of HEVs (Fig. 3A) but not SDF-1 γ (data not shown). In non-MG adults with a few HEVs, we observed that HEVs also displayed SDF-1 α/β . Analyzing mRNA

expression of *SDF-1 α* and *- β* , the two main SDF-1 isoforms expressed in the thymus (24), we did not observe significant differences in thymic extracts (Fig. S1A).

SDF-1 on HEVs can be produced by endothelial cells themselves (25) or by surrounding cells and, subsequently, transported to HEVs by transcytosis (26). To determine whether SDF-1 on thymic HEVs in MG was produced autogenously, we isolated thymic HEVs by laser-capture microdissection and analyzed the level of *SDF-1 α/β* mRNA expression by real-time PCR (Fig. 3B). For internal controls, *SDF-1 α/β* mRNA expression was evaluated in microdissected medullary thymic areas, as previous studies have described that SDF-1 is expressed by medullary thymic epithelial cells (27). We demonstrated that thymic HEVs were capable of producing *SDF-1 α/β* mRNA with expression levels 10-40 times higher than in medullary areas (Fig. 3C). Besides, on thymic sections of some non-MG adults that displayed a small number of HEVs, we also observed SDF-1 production by HEVs (data not shown). Our data thus revealed that autogenous high expression of SDF-1 is a specific characteristic of thymic HEVs.

Overall, the abnormal development of thymic HEVs expressing selectively SDF1 α/β could play an important role in thymic hyperplasia associated with MG by driving the recruitment of peripheral cells.

3.5. Peripheral cells around HEVs are partially CXCR4⁺

As SDF-1 mediates cell recruitment by binding to its receptor CXCR4, we analyzed thymic CXCR4 expression in MG. Globally, *CXCR4* mRNA expression levels did not differ between the thymus of MG patients and non-MG adults (Fig. S1B). The fact that the global CXCR4 level did not vary could be due to the dominant CXCR4 expression by TECs and thymocytes, which may mask differences between MG and non-MG adults. However, when we performed co-staining for HEVs, peripheral cells and CXCR4, we observed that some of the B cells, monocytes/macrophages, mDCs and pDCs that localized around HEVs, expressed CXCR4

suggesting that they could have been recruited through binding to SDF-1 on HEVs. We were also able to detect CXCR4⁺ monocytes/macrophages (Fig. 4B, lower row) and pDC (Fig. 4B, lower row) inside HEVs strongly supporting this hypothesis. CXCR7 is a second receptor for SDF-1, whose role in cell migration processes is debated (13, 14). Analyzing CXCR7 mRNA levels by real-time PCR, we did not observe differences between MG and non-MG thymus. By immunohistochemistry on thymic sections from MG, we only detected CXCR7 on GC-like structures (data not shown).

As we observed CXCR4⁺ peripheral cells inside and around thymic HEVs, we conclude that their recruitment to the thymus could be mediated by their interaction with SDF-1.

3.6. CXCR4 and CXCR7 levels in the periphery of MG patients

We next investigated expression levels of CXCR4 and CXCR7 in the periphery of MG patients and healthy adult controls on the main PBMC subpopulations: B cells, CD4⁺ and CD8⁺ T cells, monocytes, mDCs and pDCs. CXCR7 was only poorly expressed on cell surface of PBMCs. Comparing CXCR7 membrane and intracellular expression levels between MG patients and healthy donors, we did not detect any differences (data not shown). For CXCR4, we observed, as expected, a high expression on the surface of monocytes, pDCs and mDCs, and a lower expression on B and T lymphocytes (Fig. 5A). Comparing the expression levels between controls and MG, we noticed a significant decrease in the geometric mean of fluorescence for CXCR4 on mDCs from MG patients as well as a slight, however not significant, decrease on pDCs (Fig. 5A). The decrease in CXCR4 expression on mDCs could be related to the pathology, since it was normalized in patients under corticosteroid treatment (Fig. 5B). To determine whether this decrease was specific for CXCR4, we similarly analyzed the expression level of two other chemokine receptors known to be expressed on mDCs: CCR7 and CXCR3, the receptors for CCL19/21 and CXCL9/10/11, respectively. Comparing controls and MG, we did not observe any significant variations for CCR7, while CXCR3

expression was significantly increased on mDCs from MG patients (Fig. 5C). We next analyzed if the decrease in CXCR4 on mDCs in MG patients could be due to transcriptional regulation or increased internalization processes. Purified mDCs from patients did not exhibit a decreased *CXCR4* mRNA expression compared to mDCs from controls (Fig. S1A) nor an increased intracellular levels of CXCR4 or SDF-1 (Fig. S1B), albeit we cannot exclude that changes were masked by the large pool of intracellular CXCR4 and SDF-1. We then analyzed the number of mDCs in blood from MG patients compared to controls. We observed a significant decrease of mDCs, and also of monocytes, in MG patients which could be related to a recruitment of these cells from the periphery to the thymus (Fig. 5D). Indeed, in MG patients under corticotherapy, which reduces the number of thymic HEVs, the number of peripheral mDCs and monocytes tended to be normalized (Fig. 5E). The specific decrease in CXCR4 expression level on mDCs in MG patients might be related to a selective recruitment of CXCR4^{high} mDCs by thymic HEVs expressing SDF-1.

4. Discussion

As the thymus is the effector organ in MG and is characterized by hyperplasia, we investigated in this study the role of thymic HEVs in peripheral cell recruitment and its contribution to hyperplasia in the MG thymus. We demonstrated that thymic HEVs selectively expressed SDF-1 and that CXCR4⁺ APCs and B cells localized inside and around HEVs.

4.1. Role of thymic HEVs in MG thymus

Lymphocyte homing to SLO or inflammatory sites is mediated by interactions between circulating cells and the specialized endothelium of HEVs allowing transmigration of cells from the periphery to target tissues (9). Our study demonstrates the presence of ectopic HEVs in medullary regions, around GCs and in perivascular spaces of MG thymus and reveals a correlation between the degree of thymic hyperplasia and the number of HEVs. We also demonstrated a decreased number of HEVs in corticoid-treated patients stressing their role in MG thymic pathogenesis. We demonstrated the presence of B cells, monocytes, pDC and mDC cells in the vicinity of HEVs stressing their role in MG thymic pathogenesis. APCs located around medullary HEVs could trigger an autosensitization against AChR which is locally expressed by TECs and thymic myoid cells (28). Presentation of the AChR antigen to T cells and infiltrating B cells by APCs could thus lead to an anti-AChR response and consequently to MG. HEVs could also play a major role in sustaining the disease, as thymic hyperplasia is correlated with the anti-AChR antibody titer (7).

Ectopic HEVs and infiltrates of peripheral cells are associated with lymphoid neogenesis in inflamed tissues of various autoimmune diseases, e.g. in the joint cavity in rheumatoid arthritis, in salivary glands in Sjogren's syndrome or in the thyroid gland in Grave's disease (29), but the mechanism by which ectopic HEVs develop in inflamed tissues is not precisely known. In experimentally induced organogenesis, lymphotoxin plays a critical role in HEV

formation (30, 31). However, comparing MG patients to non-MG adults in thymic transcriptome analysis (32) or by real-time PCR (data not shown), we did not observe significant differences in lymphotoxin- α and - β mRNA expression. Studies in mice showed that overexpression of CCL21 or CXCL13 in the pancreas or thyroid gland could mediate the development of lymph node-like structures including HEVs (31, 33, 34). Interestingly, in the MG thymus, CXCL13 and CCL21 are overexpressed by thymic epithelial cells and lymphatic endothelial vessels, respectively (6, 19). These chemokines could therefore be involved in development of thymic HEVs in MG.

4.2. Role of SDF-1 on thymic HEVs in MG

Recruitment of peripheral cells to SLOs or chronically inflamed tissues is driven by the constitutive expression of adhesion molecules and chemokines at the luminal surface of HEVs (9). Searching for chemokines present on ectopic HEVs in MG thymus, we uniquely localized SDF-1 in the lumen of thymic HEVs. Even though SDF-1 can be referred to as a homeostatic chemokine, it also plays a role in several autoimmune diseases (16, 35, 36), particularly in rheumatoid arthritis, where it is similarly present on HEVs of synovial tissue (15). While immunohistochemical studies in SLOs or chronically inflamed human tissues demonstrated the presence of multiple chemokines on the surface of HEVs, mRNA encoding these chemokines were not detected suggesting that chemokines were produced by surrounding cells and transported to HEVs by a transcytosis mechanism (37-39). However, *SDF-1* mRNA expression has been detected in cultured endothelial cells isolated from rat lymph node HEVs (25). To define the origin of SDF-1 on ectopic HEVs in MG thymus, we performed laser capture microdissection and demonstrated for the first time *in situ* that thymic HEVs are able to synthesize *SDF-1* mRNA. We also detected *SDF-1* mRNA and protein expression on the few thymic HEVs in non-MG adults indicating that SDF-1 expression is an intrinsic property of ectopic HEVs in the thymus. Overall, these results suggest that the mRNA expression

profile of HEVs depends on the environment in which they develop and that each tissue affects the differentiation of HEVs in a specific way. This concept is supported by studies showing that HEV development could be induced by CCL21 overexpression in the pancreas and thyroid but not in the skin or central nerve system parenchyma (34).

4.3. Role of CXCR4 on peripheral cell recruitment in the thymus

We observed the presence of B cells, monocytes, pDCs and mDCs around ectopic HEVs in MG thymus, and some of them were clearly CXCR4-positive, indicating that they could have been attracted by SDF-1 on HEVs. Upon recruitment, these APCs could contribute to changes observed in the MG thymus. pDCs are specialized to respond to viral infections (40) by producing large amounts of cytokines and type I interferons and could therefore contribute to the characteristic interferon signature in MG thymus (32, 41). We also observed a significant decrease in monocyte numbers in blood from MG patients. The presence of monocytes around and inside HEVs suggests a peripheral recruitment. Once monocytes enter their target tissue, they can differentiate into both macrophages and mDCs. While macrophages were found to be overrepresented in the hyperplastic thymus of MG (23), where they act as a source for B-cell survival factors such as BAFF and APRIL (42), further implication in MG has never been clearly studied. For mDCs, Xia *et al.* described an abnormal number and distribution in the MG thymus (43) and Nagane *et al.* showed that mDCs localized around blood vessels and around GCs (44). In this study, we observed a significant decline of mDCs number in the blood of MG patients as well as a decreased CXCR4 expression on mDCs, which could be related to the migration of a subpopulation of peripheral mDCs expressing high levels of CXCR4 towards MG thymus via SDF-1⁺ HEVs. Likewise, Cravens *et al.* suggested that SDF-1 in the salivary glands of Sjogren's syndrome selectively recruits a CXCR4-positive subset of blood mDCs (45). Dinther-Janssen *et al.* also observed a subset of mDCs around HEV-like vessels in rheumatoid arthritis (46). The fact that the number of

mDCs and the level of CXCR4 on mDCs in the periphery tends to be normalized in patients under corticosteroid treatment, which reduces the number of thymic HEVs, favors the hypothesis that peripheral mDCs enter the MG thymus via HEVs.

5. Conclusion

In this study, we describe that HEV development is a typical feature of thymic hyperplasia in MG. Since thymic HEVs selectively express SDF-1, they could play a crucial role in peripheral cell recruitment, in particular of B cells and APCs such as monocytes/macrophages, pDCs and mDCs which could sustain the inflammatory status of the thymus and thus the autoimmune response. So far, several chemokines have been associated with thymic pathogenesis in MG. Increased thymic expression of CXCL10 was proposed to recruit peripheral CD4⁺ T cells (47) and CXCL13, a B-cell chemoattractant, was shown to be overexpressed on MG TECs (19). Overexpression of CCL21 in MG thymus was attributed to lymphatic vessels where it could regulate the import or export of circulating cells (6). Here we show that SDF-1 could be another player in the orchestra of chemokines that regulate cellular trafficking in MG thymus and thus mediate and/or maintain thymic hyperplasia. As Anti-AChR antibody titers correlate with the degree of thymic hyperplasia (2), therapies that target SDF-1/CXCR4 interaction and block peripheral cell recruitment to the thymus could be of major interest in the treatment of MG patients to avoid thymic hyperplasia.

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FIGURE LEGENDS

Figure 1. Development of HEVs in MG thymus. Immunohistochemical analyses of HEVs in the thymus of MG patients. (A) Thymic sections from MG patients were co-labeled in red with anti-PNAd antibody for HEVs and in green with antibodies against keratin for epithelial cells (top), CD3 for mature T cells (middle) or CD21 for GCs (bottom). (B) HEV labeling on thymic sections from non-MG adults (Ad), MG patients with low (ML) or high (MH) degree of thymic hyperplasia and MG patients under corticoid treatment (Cortico). (C) Quantification of the number of HEVs per thymic section (Ad, n=4; ML, MH and Cortico, n=5). *p* values were assessed by the Mann-Whitney test and indicated when less than 0.05.

Figure 2. Thymic HEVs are surrounded by peripheral cells. Thymic sections of MG patients were co-labeled for HEVs in red and peripheral cells in green. B cells were identified with anti-CD20 antibody (A, B), monocytes/macrophages with anti-CD14 antibody (C, D) mature mDCs with anti-DC LAMP antibody (E, F) and pDCs with anti-CD123 antibody (G, H).

Figure 3. Characterization of SDF-1 expression in the MG thymus. (A) MG thymic sections were stained with antibodies for anti-SDF-1 α/β (green) and PNAd (red) for HEVs. (B) Cryostat sections of human thymic tissues were labeled for HEVs with anti-PNAd antibody detected by peroxidase staining and counterstained with hematoxylin. Medullary regions (identified by their characteristic structures) and HEVs (dark brown color) were separately isolated by laser-capture microdissection (magnification 40x). (C) *SDF-1 α/β* mRNA was quantified in microdissected regions by real-time PCR and normalized to 28S. Graph bar represents the results obtained with three different MG patients and bars correspond to the mean of duplicate values \pm SEM.

Figure 4. CXCR4⁺ cells around HEVs in MG thymus. Co-staining of MG thymus sections with antibodies against CXCR4 (green), PNAd for HEVs (blue) and CD20 for B cells (A), CD14 for monocytes/macrophages (B), DC LAMP for mDCs (C) or CD123 for pDCs (D) (red). Arrows show double stained cells.

Figure 5. Decreased level of CXCR4 on peripheral mDCs. (A) The geometric mean of fluorescence for CXCR4 was assessed by flow cytometry on PBMCs from healthy control adults (Ad) and MG patients. Lymphocyte and monocyte gates were determined in the SSC/FSC plot. B cells (CD19⁺) and T cells (CD4⁺ or CD8⁺) were analyzed in the lymphocyte gate. Monocytes (CD14⁺), pDCs (CD14⁻CD123⁺) and mDCs (CD14⁻CD11c⁺) were analyzed in the monocyte gate. (Ad, n=8-14; MG, n=6-10), (B) CXCR4 expression on mDCs from controls, untreated and corticoid-treated MG patients (Ad and MG, n=10; cortico, n=6). (C) Expression level of chemokine receptors CCR7 and CXCR3 on mDCs (Ad and MG, n=7). (D) Percentage of PBMC subtypes in controls and MG patients (Ad, n=8-14; MG, n=6-10). (E) Numbers of peripheral mDCs (left) and monocytes (right) from controls, untreated and corticoid-treated MG patients (for mDCs: Ad, n=11; MG, n=10; cortico, n=6; for monocytes: Ad, n=12; MG, n=11; cortico, n=6). p values were assessed by the Mann-Whitney test and indicated when less than 0.05.

Figure 1

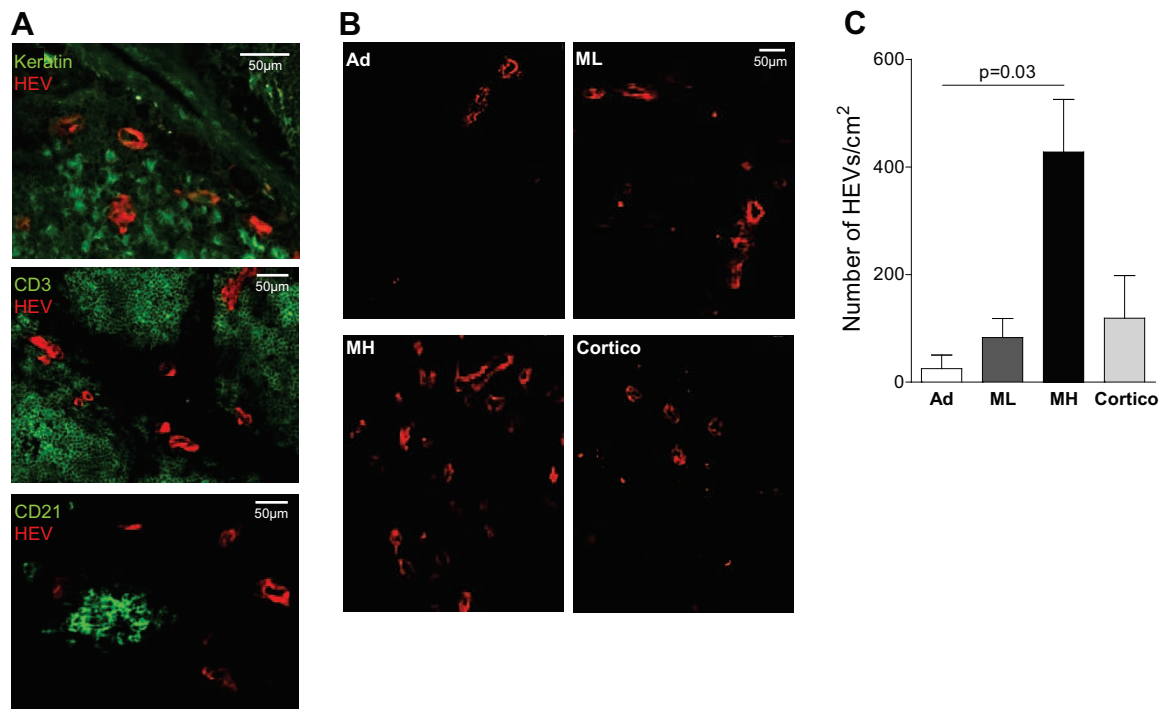


Figure 2

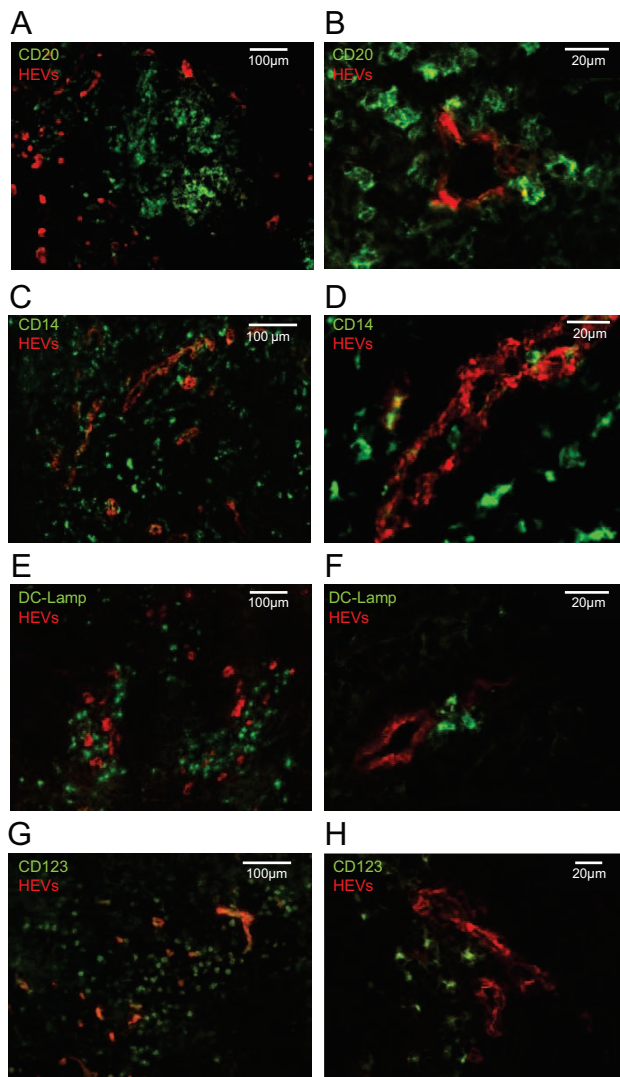


Figure 3

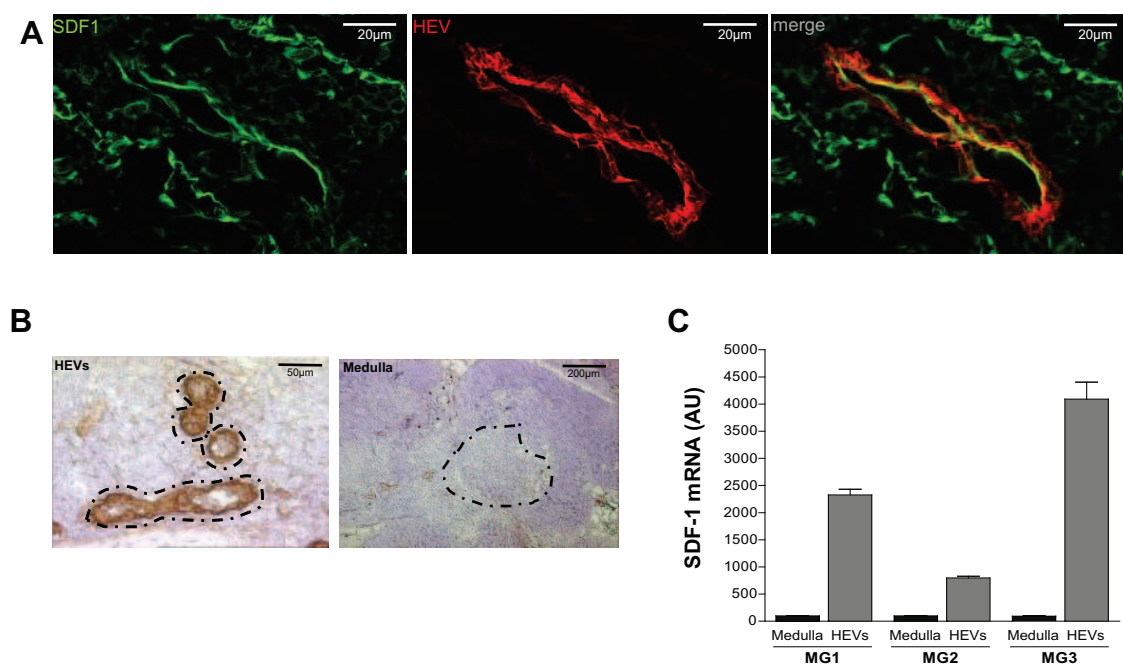


Figure 4

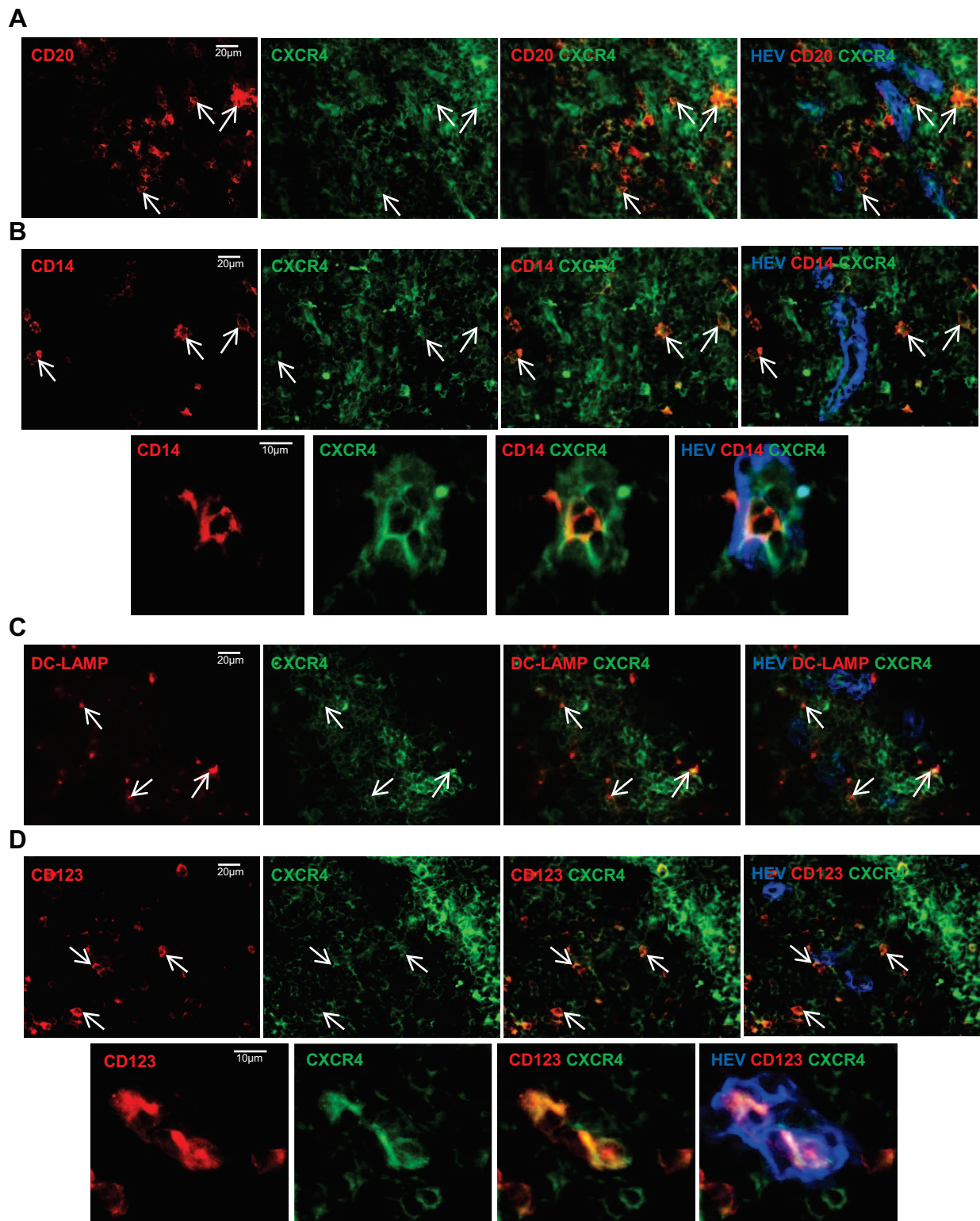
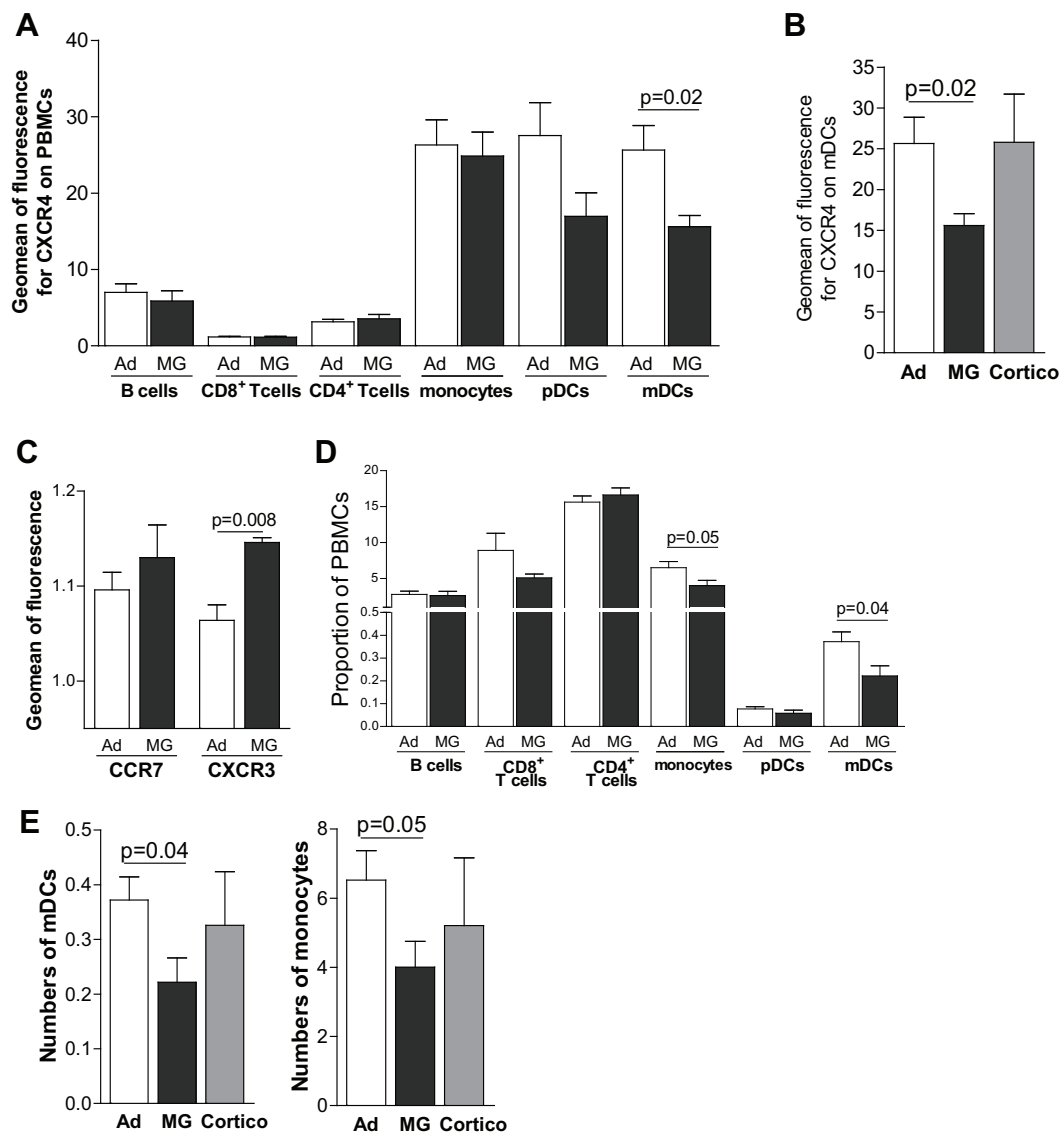
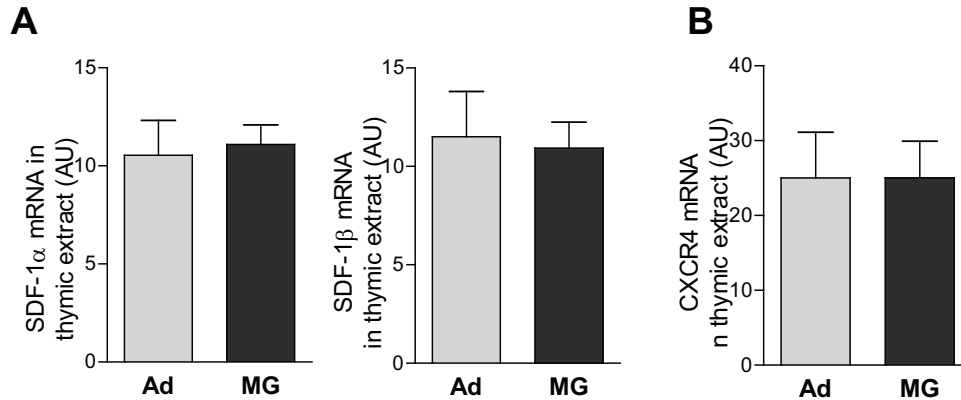


Figure 5



Supplemental figure S1



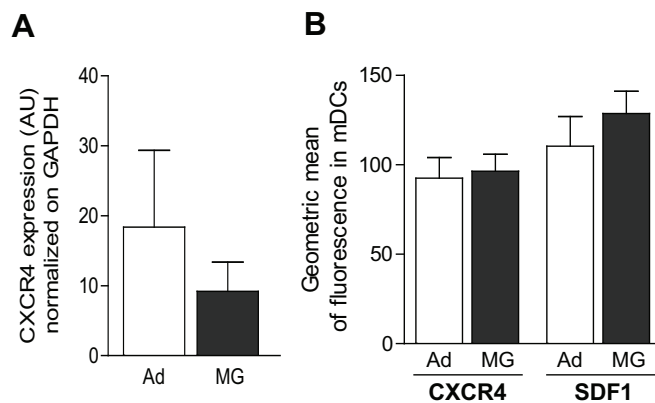
Supplemental figure 1. No difference in SDF1- α/β and CXCR4 expression between thymus from non-MG adults and MG patients. (A) Quantitative PCR analysis of SDF-1 α (left) and SDF-1 β (right) expression in thymic extracts from MG patients (n=23) and non-MG adults (n=8). (B) Analysis of CXCR4 expression in thymic extracts from MG patients (n=9) and non-MG adults (n=13). p values were assessed by the Mann-Whitney test, but none was less than 0.05.

Primer sequences were as follows:

- (1) for SDF-1 α (F:5'-CACTGTGGCTAACATTG-3', R:5'-AGGTCCTGGTGGTATT-3'),
 (2) for SDF-1 β (F:5'-TAGTCAAGTGCGTCCACGAG-3', R:5'-ACACACAGCCAGTCAACGAG-3'),
 (3) for CXCR4 (F:5'-GCCTTATCCTGCCTGGTATTG-3', R:5'-GCGAAGAAAGCCAGGATGAGG-3').

Results were normalized by GAPDH expression.

Supplemental figure S2



Supplemental figure 2. No transcriptional deregulation for CXCR4 nor modified internalization for CXCR4 or SDF-1 in mDCs from MG. (A) RNA was isolated from purified CD14-CD11c⁺ mDCs of control and MG (n=4). *CXCR4* mRNA was quantified by RT-PCR and normalized by *GAPDH*. (B) Level of intracellular CXCR4 and SDF-1 in CD14-CD11c⁺ mDCs from control adults and MG patients (n=4 and n=5, respectively).

Supplemental table S1a

List of MG patients included in the study

Patient	Age (years)	Sex	Degree of thymic hyperplasia ⁽¹⁾	Delay of onset	MGFA ⁽²⁾ score	Drugs	Anti-AChR titer (nmol/L)	Experiments
MG1	12	F	High	1 year	IV	mytelase	>100	flow cytometry
MG2	13	F	High	6 months	-	mestinon	29	IHC ⁽³⁾ , PCR
MG3	14	F	High	4 years	III	mytelase	6,6	PCR
MG4	17	M	High	1 year	II	mestinon	4,8	PCR
MG5	17	F	High	-	III	mestinon	>100	PCR, flow cytometry
MG6	18	F	High	36 years	III	mytelase	29	PCR
MG7	18	F	High	2 years	-	mestinon	33	PCR
MG8	19	F	High	1 year	III	mestinon	>100	flow cytometry
MG9	20	F	High	6 months	III	mytelase	11	IHC, PCR
MG10	21	F	High	6 months	III	mytelase	50,78	IHC
MG11	22	F	High	3 months	II	mestinon	0,6	PCR
MG12	23	F	High	1,5 years	III	mestinon	105,4	IHC
MG13	24	F	High	9 months	III	mytelase	15,8	IHC, PCR
MG14	25	F	High	3 months	II	mestinon	>100	flow cytometry
MG15	26	F	High	4 years	II	mytelase	851,9	IHC
MG16	27	F	High	2 years	II	mestinon	123,6	IHC
MG17	28	F	High	4 years	II	mytelase	9,7	IHC
MG18	28	M	High	8 years	I	mestinon	278	flow cytometry
MG19	28	M	High	> 10 years	IV	mestinon	>100	flow cytometry
MG20	32	F	High	1 year	III	mytelase	26,7	PCR
MG21	33	F	High	8 months	III	mytelase	5,1	IHC, PCR
MG22	34	F	High	7 years	-	mestinon	43	IHC, flow cytometry
MG23	36	M	High	1,5 years	III	mestinon	29	PCR
MG24	36	F	High	few years	-	mestinon	41	IHC, flow cytometry
MG25	41	F	High	6 years	III	mestinon	21,3	PCR
MG26	14	F	Low	1 year	III	mestinon	130	IHC
MG27	15	F	Low	2 months	-	metylase	36,9	IHC, flow cytometry
MG28	16	F	Low	1 month	III	mytelase	0	PCR
MG29	17	F	Low	-	-	-	37	flow cytometry, PCR
MG30	18	M	Low	3 years	I	mestinon	0	PCR
MG31	21	M	Low	1 year	I	-	0,53	flow cytometry, PCR
MG32	22	M	Low	2 years	IV	mytelase, prednisolone	2,2	IHC
MG33	22	F	Low	4 months	III	mytelase	50	PCR
MG34	23	F	Low	8 months	II	mestinon	2118,7	IHC
MG35	23	F	Low	3 months	IV	mestinon, prednisolone,	263	flow cytometry
MG36	24	F	Low	4 months	III	mytelase	1,1	IHC
MG37	24	M	Low	0,5 months	III	mytelase	1,18	flow cytometry
MG38	25	F	Low	8 years	II	mestinon, prednisolone,	66,7	IHC
MG39	25	F	Low	2 years	III	mestinon	6,6	IHC, PCR
MG40	26	M	Low	2 years	III	mestinon, prednisolone,	22,2	IHC

MG41	27	F	Low	6 years	III	mestinon	26,9	IHC
MG42	27	M	Low	5 months	II	mytelase	39	IHC
MG43	28	F	Low	2 years	II	mytelase	0,5	IHC
MG44	28	M	Low	1 year	IV	mestinon, prednisolone,	1,58	flow cytometry
MG45	28	M	Low	few months	I	mytelase, prednisolone	0,48	flow cytometry
MG46	28	F	Low	1 month	I	mestinon	0,58	IHC, flow cytometry
MG47	29	M	Low	5 months	III	mytelase	11	IHC
MG48	29	F	Low	1 year	II	mestinon	179	IHC
MG49	29	F	Low	-	I	-	83,69	flow cytometry
MG50	29	F	Low	1 year	III	mestinon	48	flow cytometry, PCR
MG51	30	F	Low	2 years	II	mestinon, prednisolone,	70,7	flow cytometry
MG52	31	M	Low	8 years	II	mytelase	26,1	IHC
MG53	32	F	Low	1 year	III	mestinon	9,9	PCR
MG54	33	F	Low	9 months	II	mytelase, prednisolone	1,5	IHC
MG55	33	F	Low	2 years	III	mestinon, prednisolone,	55,5	IHC
MG56	33	F	Low	4 years	II	mytelase	12,6	IHC
MG57	33	F	Low	4,5 months	III	mestinon	14	PCR
MG58	33	M	Low	14 years	III	mytelase, prednisolone	0,8	PCR
MG59	33	M	Low	10 months	III	-	12,6	flow cytometry
MG60	35	F	Low	-	I	-	0	PCR
MG61	36	F	Low	1,5 years	II	mestinon	123,6	IHC, flow cytometry
MG62	36	M	Low	1 year	-	mestinon, Azathioprine	34	flow cytometry
MG63	36	M	Low	-	-	-	13,6	flow cytometry
MG64	37	F	Low	8 years	I	mytelase	29,84	flow cytometry
MG65	38	M	Low	few months	III	mytelase	4	PCR
MG66	39	M	Low	2 months	-	mytelase	-	flow cytometry
MG67	40	M	Low	4 months	II	mytelase	0?	flow cytometry, PCR
MG68	42	F	Low	4 years	III	mytelase	0,38	IHC
MG69	44	F	Low	10 months	III		4,6	PCR
MG70	52	M	Low	7 months	II	mestinon	0	PCR
MG71	53	M	Low	few months	III	mytelase	0,4	PCR
MG72	27	F	-	3,5 years	-	mytelase, prednisolone	>100	flow cytometry

- (1) Degree of thymic hyperplasia: low thymic hyperplasia (with 2 or fewer germinal centers per section) or high thymic hyperplasia (with 3 or more GCs per section)
- (2) Myasthenia Gravis Foundation of America (MGFA) Clinical Classification
- (3) Immunohistochemistry
- (-) unknown

Supplemental table S1b

List of antibodies used for immunohistochemistry and flow cytometry

Antibodies for immunohistochemistry

(1) for detection and localization of HEVs

Rat IgM anti-human peripheral node addressin (PNAd) carbohydrate epitopes	553863	BD Biosciences
biotinylated mouse anti-rat IgM	550330;	BD Biosciences
Streptavidin Alexa-Fluor594	S11227	Invitrogen
Streptavidin Alexa-Fluor350	S11249	Invitrogen
mouse anti-pan cytokeratin	ab11212	Abcam
mouse anti-human CD3	F0818	Dako
mouse anti-human CD21	555421	BD Pharmingen

(2) for detection of chemokines and chemokine receptors

rabbit anti-human CXCR4	AB1846	Millipore
mouse anti-human SDF-1 α/β	MAB 350	R&D Systems
mouse anti-human SDF-1 γ	a kind gift from Dr K. Balabanian	
mouse anti-human CCL19	MAB361	R&D Systems
goat anti-human CCL21	AF366	R&D Systems
goat anti-human CXCL9	AF392	R&D Systems
mouse anti-human CXCL10	MAB266	R&D Systems
goat anti-human CXCL11	AF260	R&D Systems
goat anti-human RANTES (CCL5)	AB278NA	R&D Systems
biotinylated anti-human CXCL13	BAF361	R&D Systems
streptavidin-horseradish peroxidase	IM0309	BeckmanCoulter
DAB+ system	K3467	Dako
hematoxylin		

(3) for detection of cells around HEVs

mouse anti-human CD20	M0774	Dako
mouse anti-human DC-LAMP	donated by Schering-Plough Laboratory	
mouse anti-human CD123	555642	BD Pharmingen
mouse anti-human CD14	R0864	Dako

(4) secondary antibodies

rabbit anti-goat IgG Alexa-Fluor488	A21222	Invitrogen
goat anti-rabbit IgG Alexa-Fluor488	A11034	Invitrogen
goat anti-mouse IgG Alexa-Fluor488	A11001	Invitroge
goat anti-mouse IgG Alexa-Fluor568	A11031	Invitrogen

Antibodies for flow cytometry

(1) Identification of PBMC subpopulations

anti-human CD19 R-phycoerythrin (RPE)	R0808	Dako
anti-human CD4 RPE-Cy5	C7069	Dak
anti-human CD8 RPE	R0806	Dako
anti-human CD14 Allophycocyanin	555399	BD Pharmingen
anti-human CD14 PE-Cy7	557742	BD Pharmingen
anti-human CD11c PE	555392	BD Pharmingen
anti-human CD11c Alexa-Fluor488	301618	Biolegend
anti-human CD123 PE-Cy7	306010	Biolegend

(2) chemokine and chemokine receptors

anti-human SDF-1 Allophycocyanin	IC350A	R&D Systems
anti-human CXCR4 fluorescein isothiocyanate (FITC)	FAB170F	R&D Systems
anti-human CXCR3 FITC	FAB160F	R&D Systems
anti-human CCR7 FITC	FAB197F	R&D Systems
anti-human CXCR7-FITC	FAB42271F	R&D Systems

Supplemental table S2

List of chemokines tested for localization on HEVs by immunohistochemistry.

Chemokine (receptors)	Expression on HEVs in secondary lymphoid organs	Expression in MG thymuses
CXCL13 (CXCR5)	Human tonsils (<i>Schaerli et al., 2000</i>)	Upregulated in MG thymus (<i>Meraouna et al., 2006</i>)
CCL21 (CCR7)	Human mesenteric lymph nodes, tonsils, and Peyer patches (transcytosed) (<i>Carlsen et al., 2005</i>)	Upregulated in MG thymus (<i>Berrih-Aknin et al., 2009</i>)
CCL19 (CCR7)	Human tonsils (transcytosed) (<i>Baekkevold et al., 2001</i>)	Upregulated in MG (<i>Berrih-Aknin et al., 2009</i>)
SDF-1/CXCL12 (CXCR4 and CXCR7)	Murine lymph nodes (transcytosed) (<i>Okada et al., 2002</i>)	Increased in cortico-treated MG (<i>transcriptomic analyses</i>)
CXCL10 (CXCR3)	Murine lymph nodes (transcytosed) (<i>Nagakubo, et al., 2003</i>)	Upregulated in MG thymus (<i>Feferman et al., 2003</i>)
CXCL9 (CXCR3)	Murine lymph nodes (transcytosed) (<i>Janatpour et al., 2004</i>)	Upregulated in MG thymus (<i>transcriptomic analyses</i>)
CXCL11 (CXCR3 and CXCR7)	Unknown	Upregulated in MG thymus (<i>transcriptomic analyses</i>)
RANTES/CCL5 (CCR5)	Peripheral lymph nodes of rabbits (transcytosed) (<i>Middleton et al., 1997</i>)	Overexpressed in thymic epithelial cells in MG (<i>Colombara et al., 2005</i>)

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ARTICLE 2

Transgenic CXCL13 overexpression in the thymus promotes B-cell recruitment

OBJECTIVE

An increased number of B cells and the ectopic presence of germinal centers (GCs) are the hallmarks of the hyperplastic thymus in autoimmune MG. Since the titer of pathogenic autoantibodies in MG patient's serum correlates with the numbers of thymic GCs, it is critical to identify the factors leading to thymic hyperplasia. Our laboratory has recently found several indications that the B-cell chemoattractant CXCL13 could be involved in the enhanced recruitment of B cells in the MG thymus: 1) CXCL13 is overexpressed by thymic epithelial cells in the MG thymus; 2) Thymic extracts from MG patients have a strong chemoattractive effect on B cells. This effect is reduced when using anti-CXCL13 antibodies; 3) Thymic extracts from MG patients under corticoid treatment have a reduced effect on B-cell mobilization and a normalized serum level of CXCL13.

To examine whether an increased thymic level of CXCL13 was sufficient to mediate B-cell recruitment and to lead to thymic hyperplasia, we generated transgenic mice overexpressing CXCL13 in medullary TECs. The aim of this study was to analyze structural and cellular changes in the thymus of transgenic mice and to study their susceptibility to experimental MG (EAMG), as these mice could present a new EAMG model with thymic hyperplasia.

Transgenic overexpression of CXCL13 in the thymus promotes

B-cell recruitment

Article in preparation

Julia Miriam Weiss¹, Perrine Cufi¹, Frida Lantner², Idit Shachar², Revital Aricha², Sara Fuchs², Sonia Berrih-Aknin^{*1} and Rozen Le Panse^{*1}

¹ Unité mixte de recherche - CNRS UMR7215/INSERM U974/UPMC UM76/AIM - Thérapie des maladies du muscle strié, Institut de Myologie, 75651 Paris Cedex 13, France.

² Department of Immunology, Weizmann Institute of Science, 76100 Rehovot, Israel

*Equal contribution

Correspondence: Rozen Le Panse
Unité Mixte de Recherche
CNRS UMR7215/INSERM U974/UPMC UM76/AIM
Thérapie des maladies du muscle strié
Groupe hospitalier Pitié-Salpêtrière
105, boulevard de l'Hôpital
75651 PARIS Cedex 13 - FRANCE
Tel 0033 (0)1 40 77 81 27 Fax 0033 (0)1 40 77 81 29
rozen.lepanse@upmc.fr

Abbreviations:

AChR:	acetylcholine receptor
DC:	dendritic cell
EGFP:	enhanced green fluorescent protein
GC:	germinal center
K5:	keratin 5
MG:	myasthenia gravis
TEC:	thymic epithelial cell
Tg:	transgenic
WT:	wildtype

ABSTRACT

An increased number of B cells often associated with ectopic germinal centers (GCs) development are the hallmarks of the hyperplastic thymus in patients of autoimmune Myasthenia Gravis, a muscular disease mostly mediated by anti-acetylcholine receptor antibodies (AChR).

Recently, the B-cell chemoattractant CXCL13 was shown to be overexpressed by thymic epithelial cells (TECs) in MG and was proposed to be a key factor in thymic hyperplasia development. To study whether elevated levels of CXCL13 were sufficient to mediate B-cell recruitment and possibly to lead to thymic hyperplasia, we generated transgenic mice overexpressing CXCL13 in medullary TECs. Our data demonstrate that transgenic K5-CXCL13 mice specifically overexpressed CXCL13 in the thymus, while no other tested chemokines were upregulated. The elevated levels of CXCL13 resulted in an increased number of B cells in the thymus of transgenic mice, which localized preferentially in loose aggregates in medullary areas. We are currently investigating, if these mice are more susceptible to experimental autoimmune MG (EAMG) in order to develop a new EAMG model associated with thymic hyperplasia.

INTRODUCTION

Autoimmune myasthenia gravis (MG) is a neuromuscular disease that is mainly mediated by auto-antibodies against the acetylcholine receptor (AChR) at the neuromuscular junction [1]. In a large majority of MG patients, the thymus is the primary production site of pathogenic autoantibodies and shows typical characteristics of secondary lymphoid organ. It exhibits a hyperplasia due to ectopic germinal centers (GCs) containing anti-AChR autoreactive B and T cells [2],[3] and the abnormal presence of high endothelial venules [4],[5]. These thymic abnormalities correlate with MG pathogenesis, as the peripheral anti-AChR antibody concentration is associated with the presence of thymic GCs [6]. Thymectomy is therefore beneficial for MG patients with thymic hyperplasia and can lead to a gradual decline of the anti-AChR antibody titer [7]. Although a lot of progress has been made in understanding MG pathogenesis, the exact mechanism underlying the B-cell recruitment and the formation of GCs in MG thymus remains unclear.

Recently, the chemokine CXCL13 was shown to be involved in MG and suspected to play a central role in thymic hyperplasia development [8],[9],[10]. CXCL13 is a constitutive chemokine expressed by stromal cells in lymph nodes (LNs), spleen or thymus where it can attract naive B cells by interacting with its receptor CXCR5 [11]. Mice deficient in CXCL13 fail to form lymphoid follicles and are characterized by malformed Peyer's patches and a lack of inguinal LNs. Experiments in mice showed that ectopic expression of CXCL13 in non-lymphoid tissue was sufficient to induce development of LN-like structures including B and T cell zones and high endothelial venules [12]. Due to its role in B-cell recruitment and lymphoid tissue organization, CXCL13 is implicated in a wide range of autoimmune disorders characterized by B-cell activation and GC formation [13],[14],[15]. In MG patients with thymic hyperplasia, CXCL13 was shown to be overexpressed by thymic epithelial cells (TECs) and to be reduced in patients under cortico-therapy in correlation with clinical

improvements [9]. Anti-CXCL13 antibodies blocked the chemoattractive effect of thymic extracts on B cells suggesting that CXCL13 could be responsible for the large B-cell infiltration into the MG thymus and thus for the establishment of thymic hyperplasia. To study whether CXCL13 was sufficient to mediate B-cell recruitment and possibly to lead to thymic hyperplasia, we generated transgenic (Tg) mice overexpressing CXCL13 in TECs. Thymic CXCL13 overexpression was found to be sufficient to provoke B-cell infiltration and the formation of loose B cell aggregates in the thymus of Tg mice. These mice could therefore present the basis for a new animal model for EAMG with thymic hyperplasia.

RESULTS

1 – Thymic overexpression of CXCL13 in Tg K5-CXCL13 mice.

We generated a line of Tg mice that carries the gene for murine CXCL13 under the control of the keratin 5 promoter, in order to mimic the overexpression of CXCL13 in medullary epithelial cells of the MG thymus. Compared to age-matched wildtype (WT) mice, K5-CXCL13 Tg mice did not show any obvious anomalies concerning body or thymus weight (data not shown).

By real-time PCR, we first analyzed CXCL13 mRNA expression in the thymus of 6-week-old heterozygous K5-CXCL13^{+/-} Tg mice. Figure 1A shows that, in very young mice the levels of CXCL13 in Tg mice were up to 30 times higher than in age-matched WT mice.

Next, we carried out the same analyses on homozygous K5-CXCL13^{+/+} mice of 3, 5 and 10 months in order to investigate whether CXCL13 expression in the thymus changes with age (Figure 1B). The difference in thymic CXCL13 expression between WT and K5-CXCL13^{+/+} mice was most prominent in young mice of 3 months with a 10-fold increased CXCL13 expression level compared to WT mice but less than the one observed on 6-week-old mice. While the CXCL13 level in the thymus increased with age in both WT and K5-CXCL13^{+/+} mice, the difference between WT and Tg mice diminished (Figure 1B). When we analyzed the CXCL13 expression in the spleen, which does not express keratin 5, we did not observe significant differences between WT and K5-CXCL13^{+/-} or K5-CXCL13^{+/+} mice (Figure 1C-D).

These data attest that we succeeded to generate a new Tg mouse line, which overexpresses CXCL13 in the thymus. This overexpression is especially important in young mice and less obvious in ageing animal, as thymic CXCL13 expression increases with age in both WT and K5-CXCL13^{+/+} mice.

2 – Expression level of other chemokines in the thymus of K5-CXCL13 mice

To examine whether the expression of other chemokines was altered in the thymus of K5-CXCL13 mice, we investigated chemokines that are known to be expressed in the thymus and that are dysregulated in the hyperplastic thymus of MG patients: SDF-1 [5], CXCL10 [16], CCL21 and CCL19 [8], [4]. Analyzing their mRNA level by real-time PCR, we did not observe any obvious change between WT and K5-CXCL13^{+/+} mice. In general, the expression of CCL21 and CCL19 in the thymus of WT and Tg mice increased with age, while CXCL10 expression levels were stable over time. Our data reveals no evident modification of the expression of other chemokines than CXCL13 in the thymus of K5-CXCL13 mice.

3 – Analysis of the proportion of thymic cells in K5-CXCL13 mice

To determine if the CXCL13 overexpression influences the cellularity in the thymus, we compared the absolute number of thymic cells between WT and K5-CXCL13^{+/+} mice. Figure 3A shows that at the age of 3 and 5 months, the number of cells was higher in the thymus of Tg mice than in WT mice, whereas no difference was observed in 10-month-old mice. This increase in the total number of thymic cells in young Tg mice suggests an enhanced migration of cells into the thymus and/or a defect in cell export out of the thymus.

To assess if thymopoiesis could be affected in Tg mice, we analyzed the proportion of CD4 and CD8 thymic subpopulations (Figures 3B-F). For CD4 single positive, CD8 single positive and CD4⁻CD8⁻ double negative T cells, the percentage was decreased in the thymus of Tg mice compared to WT mice, whatever the age. However, for CD4⁺CD8⁺ double positive T cells, the percentage was slightly augmented in Tg compared to WT mice. The slight changes regarding the proportion of CD4⁺CD8⁺ thymocytes could reflect perturbations in the differentiation process of T cells but require further investigations.

As CXCL13 acts especially as a B-cell chemoattractant, we analyzed the proportion of B cells in the thymus by flow cytometry using the pan-B cell marker CD19. In parallel, we

determined the proportion of natural killer cells (NK1.1 marker), which were mobilized to the gut in a Tg mouse model overexpressing CXCL13 in the intestine [17], as well as monocytes/macrophages (CD11b marker), since Weiss *et al.* had observed their recruitment to the MG thymus [5]. Flow cytometry analysis did not reveal increased percentages for these cell populations in the thymus in Tg mice (Supplemental figure S2). Surprisingly, we even observed a slight decrease.

4 – Analysis of B cells in the thymus of K5-CXCL13 mice

As analyses by flow cytometry did not allow us to detect differences in the numbers of thymic B cells between WT and Tg mice, we investigated the expression of the B-cell marker B220 by real-time PCR. Figure 2A shows that the B220 levels were increased in the thymus of 6-week-old K5-CXCL13^{-/+} mice as well as in K5-CXCL13^{+/+} mice of 5 and 10 months compared to age-matched WT mice. For 3-month-old K5-CXCL13^{+/+} mice, we observed a slight and not significant decrease in B220 expression (Figure 4B). In the spleen, B220 levels did not differ significantly between WT and K5-CXCL13^{+/+} Tg mice (Figure 4C-D).

In parallel, we analyzed the distribution of B cells in the thymus by immunofluorescent staining. Thymic sections of WT and K5-CXCL13^{-/+} mice were co-stained with an anti-B220 antibody to detect B cells and an anti-keratin antibody to label the epithelial network of the thymus (Figure 5A and B). To quantify thymic B cells, sections were divided in equal fields and the number of B cells was determined in each field (Figure 5C). The staining revealed that B cells were more abundant in the thymus of Tg mice than in WT mice and that B cells localized primarily in medullary regions. While we were not able to detect GCs, we observed spots, where B cells loosely accumulated. Similar analyses to assess the presence of B cells in the thymus of homozygous K5-CXCL13^{+/+} mice are in progress. Overall, our preliminary results suggest that thymic overexpression of CXCL13 induces a recruitment of B cells to the thymus. The discrepancy between results obtained by flow cytometry and by

immunohistochemistry/PCR could be due to technical issues. A simple, mechanical homogenization may not be sufficient to isolate a maximum of B cells from the thymus. Wilcox *et al.* had shown that thymic B cells are recovered more efficiently by enzymatic digestion than by conventional dispersion [18].

DISCUSSION

Thymic hyperplasia in MG is associated with the dysregulation of several chemokines [6]. Especially CXCL13, a well-known B cell chemoattractant, seems to play a key role in thymic hyperplasia establishment, as previous results had shown that CXCL13 expression was upregulated in TECs of MG patients and its reduction correlated with clinical improvements [9]. Thymic extracts of MG patients had a strong chemoattractive effect on B cells that was inhibited when using anti-CXCL13 antibodies [9]. We thus wanted to test as a proof of principle if thymic overexpression of CXCL13 would induce ectopic B-cell recruitment, leading eventually to thymic hyperplasia. In this study, we therefore generated Tg mice overexpressing CXCL13 in medullary TECs. We were able to show that the overproduction of CXCL13 in Tg mice induced an augmentation of the cell number in the thymus. Analysis by immunohistochemistry and PCR revealed that the thymus contained increased numbers of B cells, which localized in loose aggregates in medullary areas. The enhanced recruitment of B cells seems specifically due to CXCL13, as no other chemokine were overexpressed in the thymus of Tg mice.

Apart from the recruitment of B cells, we observed an increased proportion of $CD4^+CD8^+$ cells in the thymus of K5-CXCL13 mice. So far, no reports exist that assign a role in thymopoiesis to CXCL13 or to its receptor CXCR5. The fact that numbers of $CD4^+CD8^+$ thymocytes were elevated in the thymus of K5-CXCL13 mice suggests that a CXCL13 overexpression could accelerate the differentiation of $CD4^-CD8^-$ thymocytes into $CD4^+CD8^+$ thymocytes or inhibit the differentiation of $CD4^+CD8^+$ thymocytes into single positive T cells. Studies investigating the ratios of thymocytes in the thymus of MG patients demonstrated a decreased percentage of $CD4^+CD8^+$ thymocytes [19],[20]. It is also possible that the changes in the proportions of thymocytes is not a direct effect of elevated CXCL13 levels, but rather an indirect effect due to other factors induced by CXCL13, for instance the increased number

of B cells could have an effect on thymocyte development. A recent study of Akirav *et al.* has demonstrated that thymic B cells can regulate the expression of certain tissue specific antigens by TECs via the production of lymphotoxin (LT) α and LT β and thus participate in the thymic selection processes [21]. Analyzing the expression of LT α and LT β in the thymus of K5-CXCL13, we indeed observed increased levels in mice of 5 months (data not shown).

Ectopic expression of CXCL13 in different organs has been induced in other Tg mouse models and also mediates cell infiltration and accumulation of B cells. CXCL13 overexpression in β cells of the pancreatic islets lead to the formation of LN-like structures that contained B and T cell zones, development of high endothelial venules, and elevated levels of CCL21 [12]. In a recent study, where CXCL13 was expressed under the control of the villin promoter in intestinal epithelial cells to mimic gut inflammatory conditions, the increased CXCL13 levels favored mobilization of B cells and of natural killer cells [17].

Currently, immunohistochemical analyses of thymic B cells in homozygous K5-CXCL13 mice of different age are in progress. Preliminary results confirm the presence of B cells within the thymus of Tg mice, albeit we did not observe GC organization (data not shown). Besides, we did not observe any obvious MG symptoms in these mice whatever their age. The formation of GCs and the induction of MG in K5-CXCL13 Tg mice might therefore require (an) additional(s) factor(s). In this context, the application of Poly(I:C), a synthetic analog of double-stranded RNA, could be helpful. A recent study in our laboratory demonstrated that intraperitoneal injections of mice with Poly(I:C) elevated α -AChR expression in the thymus and resulted in the production of anti-AChR antibodies in the periphery [22]. The administration of Poly(I:C) to K5-CXCL13 mice could lead to an increased expression of thymic AChR subunits in an environment that favors the recruitment of B cells. This combination could be sufficient to mediate thymic hyperplasia with an anti-AChR immune response and hence MG. This hypothesis needs to be evaluated.

Currently, most of the MG research in animals depends on an immunization with purified AChR, which results in induced experimental autoimmune MG (EAMG). EAMG animals develop similar features than MG patients including muscle weakness, presence of serum anti-AChR antibodies and loss of muscle-AChR [23],[24],[25],[26]. One main drawback of induced EAMG models is that animals never exhibit thymic hyperplasia [27]. As the antigen is directly injected, it may bypass the initial and sustaining events involved in human MG that seem to involve the thymus. Therefore, we are currently testing the EAMG model using our Tg K5-CXCL13 mice. We intend to analyze the susceptibility of these mice to MG compared to the classical EAMG model and to investigate if the animals present signs of thymic hyperplasia. An EAMG model with thymic hyperplasia would be very helpful to test new therapeutic approaches that block CXCL13 or other factors implied in thymic hyperplasia development to specifically abrogate the B-cell recruitment to the thymus.

MATERIALS AND METHODS

Vector construction and verification

A keratin 5 (K5) promoter driven mouse CXCL13 transgene (K5-CXCL13) was prepared in order to obtain CXCL13 expression in medullary TECs. A pEYFP 1 plasmid expressing the enhanced green fluorescent protein (EGFP) gene under the bovine K5 promoter was kindly provided by Prof Daniel Aberdam (INSERM U898, Nice, France). The EGFP gene was then replaced with the full-length cDNA of mouse CXCL13 by the cloning service of GeneCust Europe (Dudelang, Luxembourg) (Supplemental figure S1A). Functionality of K5-mCXCL13 vector was verified by transfection of the human epithelial cell line HaCat known to express keratin 5. Two days after transfection, cells were analyzed for CXCL13 at the mRNA and protein level. We demonstrated by PCR, ELISA and immunohistochemistry that transfected cells expressed murine CXCL13, which confirmed the functionality of the K5'mCXCL13 plasmid (Supplemental figure S1B-D).

Transgenesis

For microinjection, the K5-CXCL13 transgene flanked by Not1 restriction site was separated from the vector by digestion with restriction enzymes Not1 (Promega, Charbonnieres, France) followed by gel-separation and purification with QIAquick gel extraction kit (Invitrogen, Villebon sur Yvette, France). The size of the isolated construct was verified by gel-electrophoresis.

The injection of the linearized K5-CXCL13 transgene into fertilized oocytes from C57BL/6 mice was performed according to the standard protocol of the transgenesis facility of the Weizmann institute (Rehovot, Israel). About 200 microinjected zygotes were transferred to 20 pseudo-pregnant females resulting in a founder mouse which was backcrossed to C57BL/6 WT mice. The transgene was detected by classical and quantitative PCR on DNA extracted from tail tissue using the following primers: 5'- GCTGAAGTCCCTGAAGCAAG (K5,

forward) and GTATTCTGGAAGCCCAT (CXCL13, reverse). Homozygote mice in the third and fourth generation with high levels of K5-CXCL13 transgene were identified by quantitative PCR. Homozygosity was confirmed by crossing between Tg and WT mice resulting in offsprings, which were all Tg.

Flow Cytometry

Mice were sacrificed by CO₂ inhalation, and the blood was obtained by cardiac punctation. Single cell suspensions from thymus and spleen were prepared by passing the organs through a nylon mesh. For spleen and blood samples, erythrocytes were removed using lysis buffer from BD Bioscience (Le Pont de Claix, France). All isolated cells were then incubated for 5 minutes on ice with Fc-block (BD Bioscience) to reduce unspecific binding of antibodies to Fc-receptor. Upon blockage, cells were incubated for 30 minutes on ice with anti-CD19 PE (553786), anti-CD4 Alexa700 (557956), anti-CD8a PE-Cy7 (552877) antibodies from BD Bioscience.

Thymic cell number

To assess the number of cells in the thymus, we weighed the entire thymus and then separated and weighed a part of it. We then counted the cells in this separated part by flow cytometry cell counting beads (BD Bioscience, 3404876) and projected the number of cells to the whole thymus.

Immunohistochemistry

Cryosection of thymic samples (7µm) were collected on superfrost slides (Thermo Fisher Scientific, Braunschweig, Germany), fixed in ice-cold acetone for 20 minutes and blocked with 2% BSA to avoid unspecific binding. Sections were stained with anti-mouse pan Cytokeratin-FITC antibody (ab78478, abcam, Paris, France) to label the epithelial network,

while B cells were detected with a biotinylated anti-B220 antibody (553085, BD bioscience) followed by staining with streptavidin Alexa-Fluor594 (S11227, Invitrogen). Images were acquired with a ZeissAxio Observer Z1 Inverted Microscope.

PCR

Total RNA was extracted as previously described [28]. 1µg of RNA was reverse transcribed for 1h at 42°C using AMV (Roche Applied Science, Mannheim, Germany) with oligo-dT (Invitrogen). Real-time PCR reactions were performed with the LightCycler® 480 Real-Time PCR System as previously described [28][1]. The primer sequences (Eurogentec, Angers, France) were as follows: (1) for murine CXCL13 (F: 5'-TGAGGCTCAGCACAGCAA-3'; R: 5'-ATGGGCTTCCAGAATACCG-3'), (2) for B220 (F: 5'- GGGTTGTTCTGTGCCTTGTT-3'; R: 5'-GGATAGATGCTGGCGATGAT-3'), (3) for murine GAPDH (F: 5'-AACTTTGGCATTGTGGAAGG-3'; R: 5'-ACACATTGGGGGTAGGAACA-3'). All samples were normalized to GAPDH.

Statistical analyses

In bar graphs, results are expressed as means of different experiments or duplicate values. Error bars represent SEM. For 2-by-2 comparisons, non-parametric Mann-Whitney test was applied as specified in figure legends.

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FIGURE LEGENDS

Figure 1. CXCL13 overexpression in K5-CXCL13 mice.

CXCL13 expression was assessed by real time-PCR. CXCL13 mRNA level in the thymus (A) and spleen (C) of wildtype (WT) and heterozygous K5-CXCL13^{-/+} transgenic mice (tg^{-/+}) of six weeks (n=2 per category). CXCL13 mRNA expression in WT and homozygous K5-CXCL13^{+/+} mice (tg^{+/+}) at different age in the thymus (B) and the spleen (D) (WT, n=4-6 and K5-CXCL13, n=3-4). PCR results were normalized to GAPDH.

Figure 2. mRNA expression level of other chemokines.

Analysis of SDF-1 (A), CXCL10 (B), CCL21 (C) and CCL19 (D) expression in the thymus of wildtype (WT) and homozygous K5-CXCL13^{+/+} (tg^{+/+}) mice by real time-PCR. (WT, n=4-6 and K5-CXCL13, n=3-4). PCR results were normalized to GAPDH.

Figure 3. Proportion of thymocytes in homozygous transgenic mice.

(A) Absolute number of cells in the thymus of wildtype (WT) and homozygous K5-CXCL13 mice (tg^{+/+}). (B) Representative flow cytometry dot plot of CD4 vs CD8 staining. The graph blots show proportions of double negative thymocytes (C), double positive thymocytes (D), CD4 single positive (SP) T cells (E) and CD8 SP T cells (F) in the thymus of WT and K5-CXCL13^{+/+} mice. (wt, n=4-7; hom-tg, 3-4).

Figure 4. B220 overexpression in K5-CXCL13 mice.

Quantitative PCR analysis of B-cell marker B220 in the thymus (A) and spleen (C) of wildtype (WT) and heterozygous K5-CXCL13^{-/+} transgenic mice (tg^{-/+}) of six weeks (n=2 per category). B220 mRNA expression in WT and homozygous K5-CXCL13^{+/+} mice (tg^{+/+}) at

different age in the thymus (B) and the spleen (D) (WT, n=4-6 and K5-CXCL13, n=3-4). PCR results were normalized to GAPDH.

Figure 5. B-cell recruitment to the thymus of K5-CXCL13 mice.

Thymic sections from wildtype (A) and K5-CXCL13^{-/+} transgenic (B) mice were stained with anti-pan Keratin antibody in green and anti-B220 antibody in red. (C) Quantitative analysis of thymic B cells in wildtype (WT) and K5-CXCL13^{-/+} (tg) mice. Sections were divided in equal fields and number of B cells was assessed in each field.

Figure 1

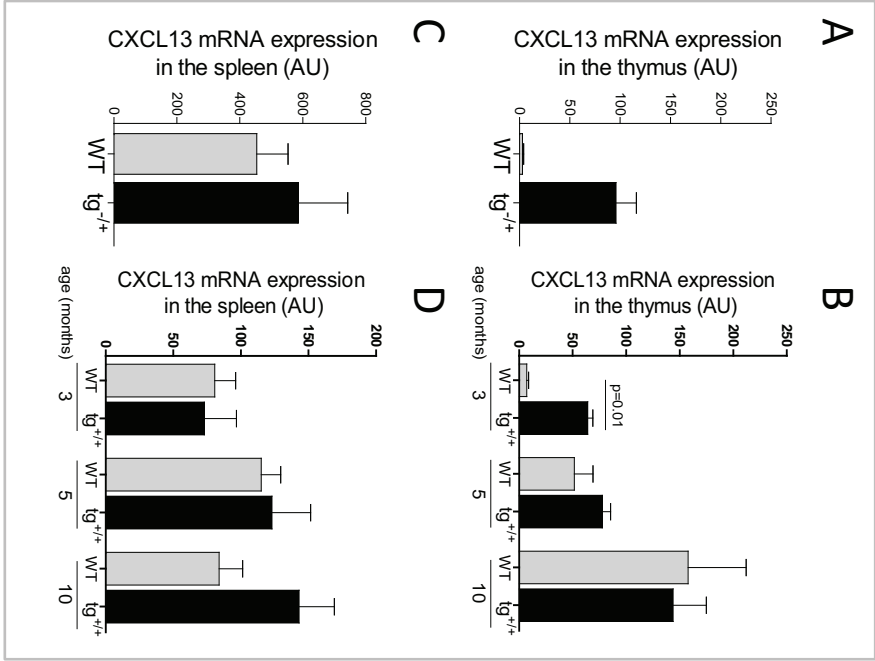


Figure 2

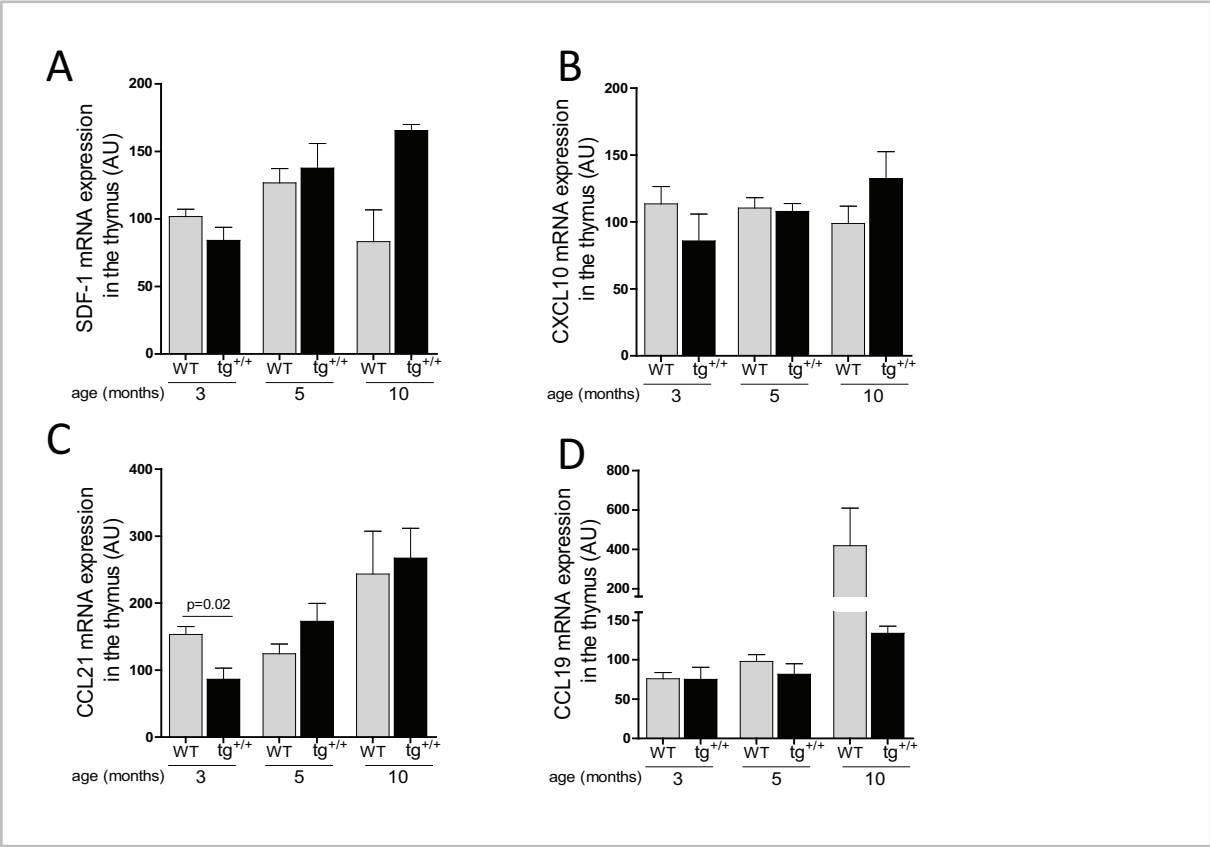


Figure 3

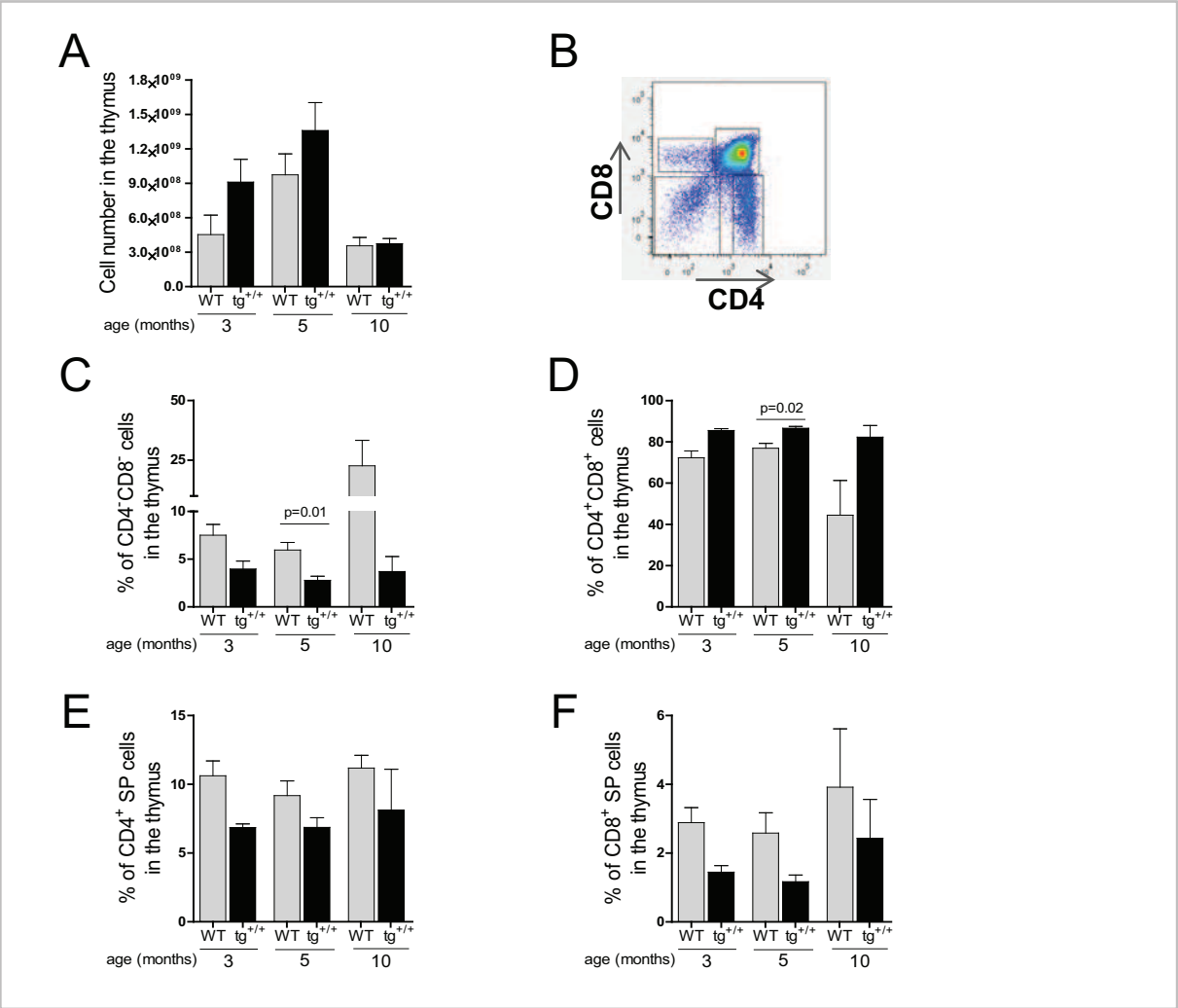


Figure 4

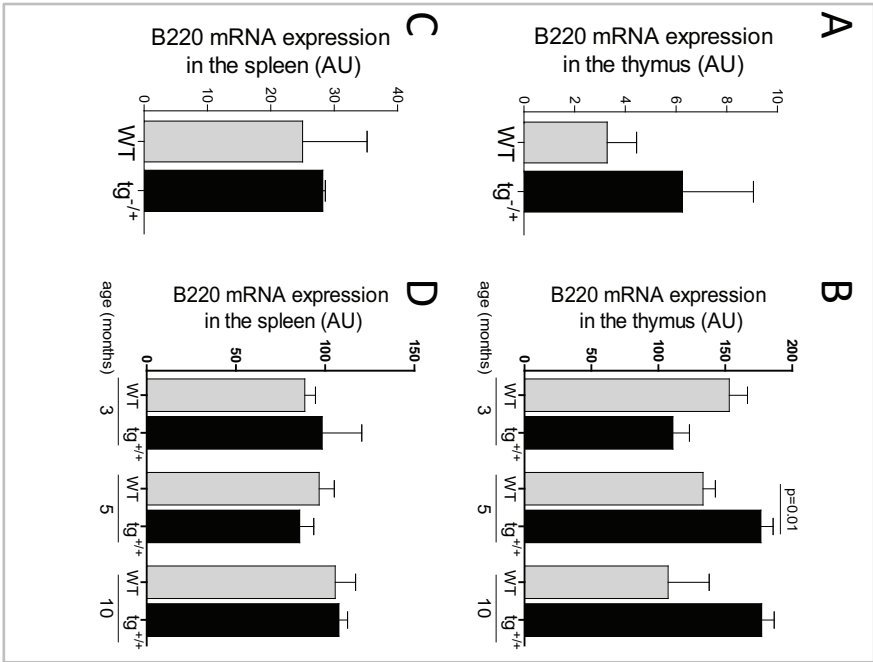
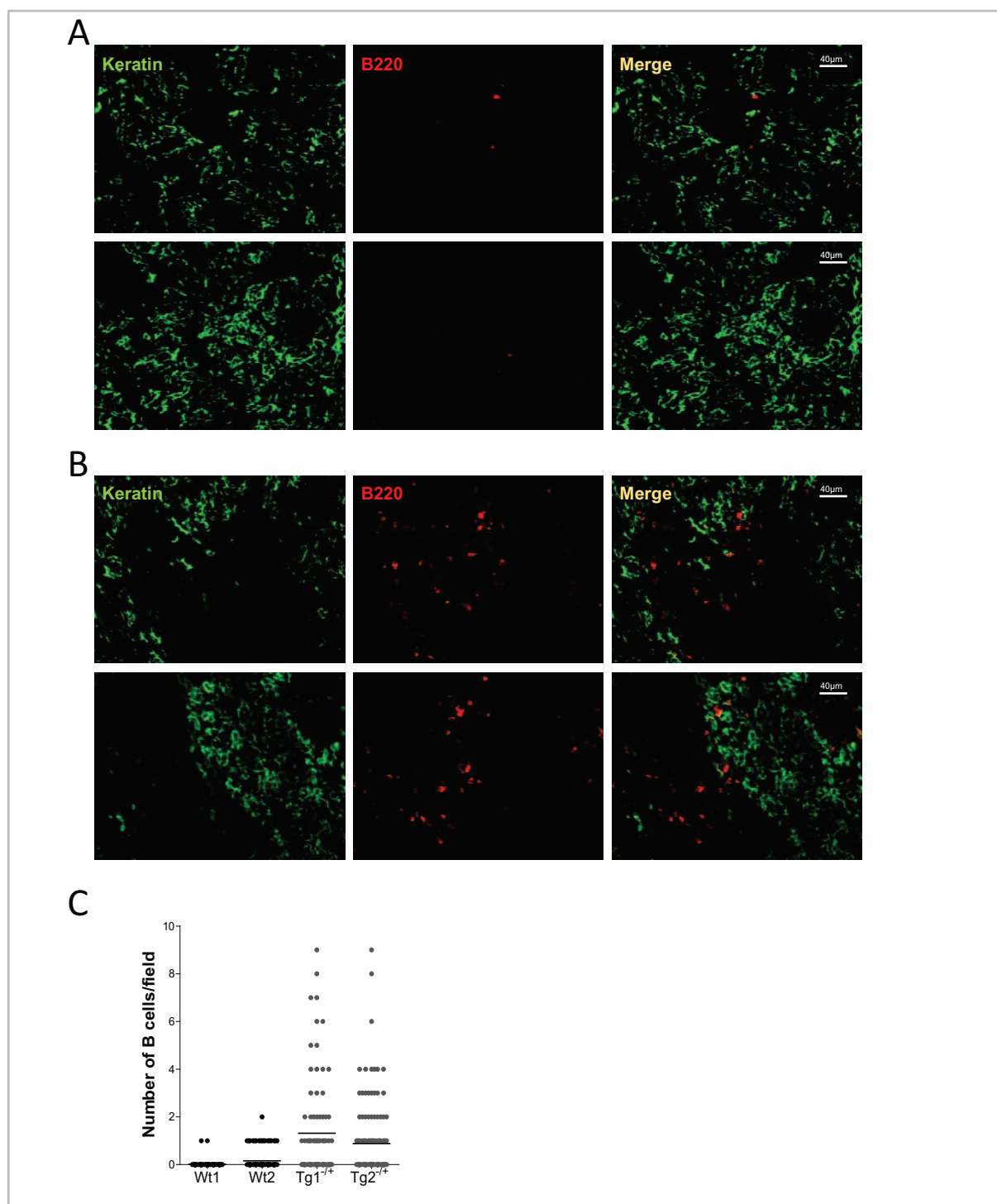


Figure 5



Supplemental figure S1

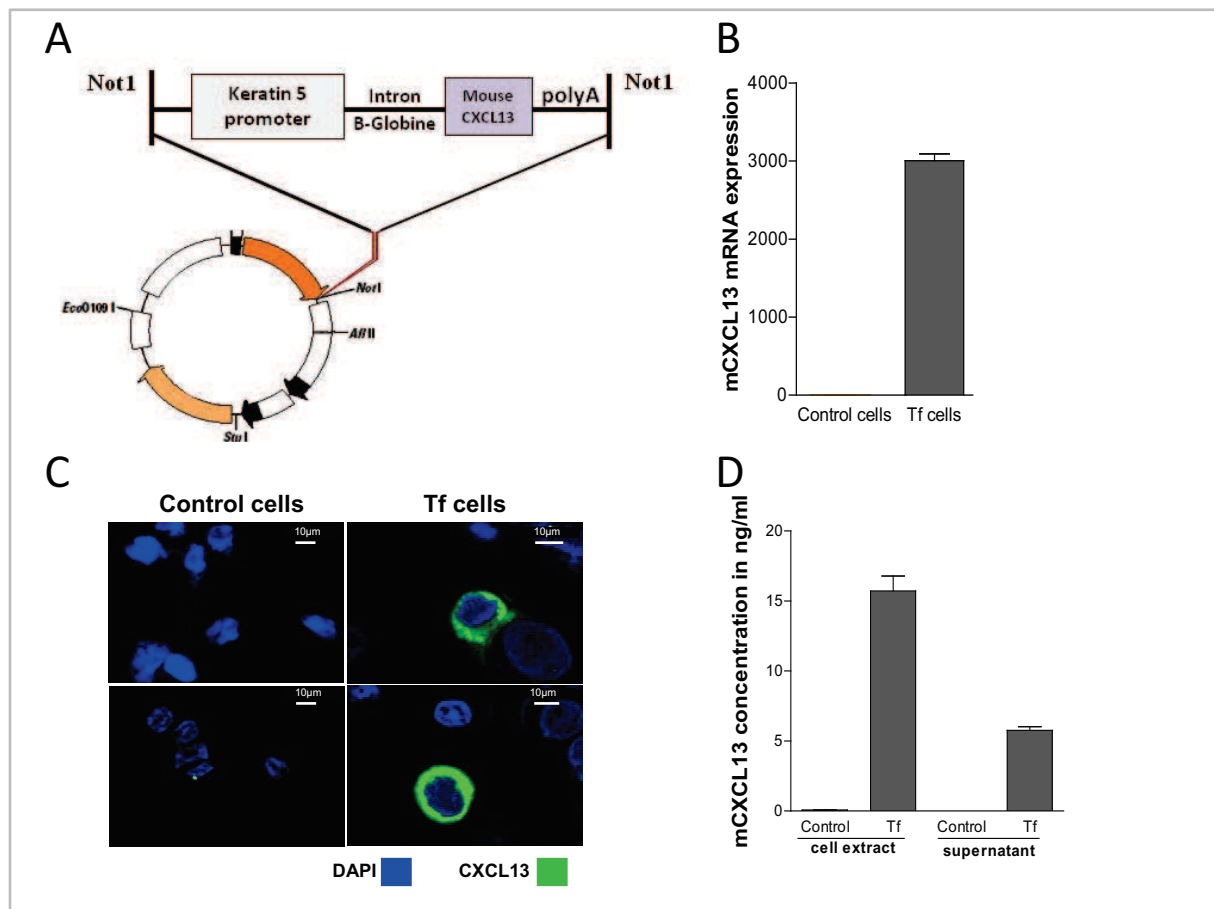


Figure S1. K5-CXCL13 vector leads to Keratin 5 driven CXCL13 expression. Figure A shows a scheme of the EYFP-1 plasmid which carries the bovine K5 promoter sequence, β -globuline introns and a poly-A termination sequence. Functionality of the K5-CXCL13 vector was verified by transfection of the keratin 5 expressing human cell line HaCat. The plasmid (500 ng) was introduced into the cells by lipofection according to the jetPEI protocol (Polyplus-transfection SA, Illkirch, France). For negative controls, HaCat cells were treated with the transfection reagents alone. Two days later, control and transfected (tf) cells were harvested and CXCL13 expression was analyzed on mRNA level by PCR (B) and on protein level by immunohistochemistry (C) and by ELISA (D). The latter was performed on both cell extract and cell supernatant.

Supplemental figure S2

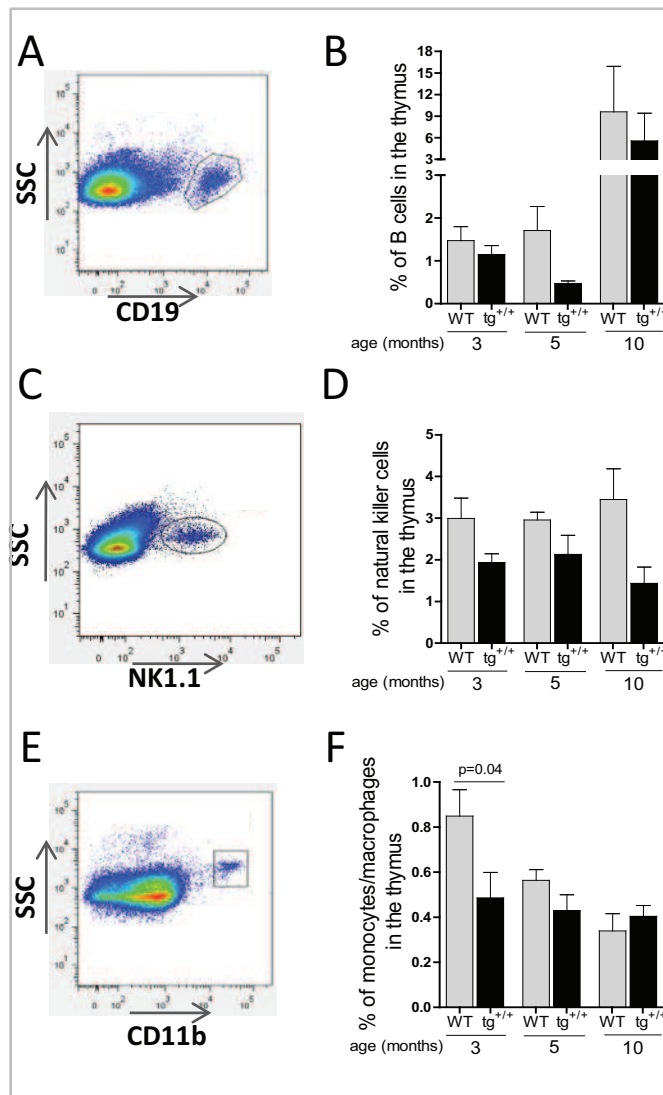


Figure S2. Proportion of thymic subpopulations in the thymus of homozygous K5-CXCL13 mice. Representative flow cytometry dot plot of B cells, natural killer cells and monocytes/macrophages identified by CD19 (A), NK1.1 (B) and CD11b staining (C), respectively. Figure 3 B, D and F show their percentages in the thymus of wildtype (WT) and homozygous K5-CXCL13^{+/+} (tg^{+/+}) mice at different age. (WT, n=4-6 and K5-CXCL13, n=3-4)

ARTICLE 3

Implication of dsRNA signaling in the etiology of autoimmune myasthenia gravis

OBJECTIVE

The hyperplastic thymus of MG patients is a chronically inflamed tissue, which shows the features of a tertiary lymphoid organ including cell infiltration, neoangiogenic processes and overexpression of inducible chemokines such as CXCL13 and CCL21. As the inflammatory thymus of MG patients is the major production site of autoantibodies against AChR, we are investigating the events that initiate the inflammatory environment. Recent results from our group have revealed an IFN-I signature in the MG thymus and demonstrated that IFN-I was able to induce a thymic overexpression of CXCL13 and CCL21. We therefore studied the factors that could trigger IFN-I production in the thymus. As IFN-I is involved in antiviral responses, we analyzed the effect of Toll-like receptor (TLR) agonists, which mimic pathogen-associated molecules, on thymic epithelial cells. From all the TLR ligands tested, we observed a specific effect of Poly(I:C), a synthetic analogue of dsRNA. Poly(I:C) treatment of thymic epithelial cells did not only trigger IFN-I signaling but also induced the expression of α -AChR. We therefore studied the role of dsRNA signaling pathways in triggering inflammation and anti-AChR sensitization in the thymus.

Implication of dsRNA signaling in the etiology of autoimmune myasthenia gravis

Perrine Cufi¹, MSc, Nadine Dragin, PhD^{1*}, Julia Miriam Weiss, MSc^{1*}, Pilar Martinez-Martinez, PhD², Régine Roussin, MD³, Sonia Berrih-Aknin, PhD¹ and Rozen Le Panse, PhD¹

¹ Unité mixte de recherche - CNRS UMR7215/INSERM U974/UPMC UM76/AIM - Thérapie des maladies du muscle strié, Groupe hospitalier Pitié-Salpêtrière, 105 Boulevard de l'Hôpital, 75651 PARIS Cedex 13 - France.

² Department of Neuroscience, School of Mental Health and Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University, Universiteitssingel 50, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

³ Department of Pediatric Cardiac Surgery, Hôpital Marie Lannelongue, 92350 Le Plessis Robinson, France.

* These authors contributed equally to this work.

Correspondence: Rozen Le Panse
UMR - CNRS UMR7215/INSERM U974/UPMC UM76/AIM
Thérapie des maladies du muscle strié
Groupe hospitalier Pitié-Salpêtrière - 105, boulevard de l'Hôpital
75651 Paris Cedex 13 - FRANCE
Tel 0033 (0)1 40 77 81 27 Fax 0033 (0)1 40 77 81 29
rozen.lepanse@upmc.fr

Running head : dsRNA signaling in autoimmune myasthenia gravis

Abbreviations: AChR: acetylcholine receptor
AIRE: autoimmune regulator
EAMG: experimental autoimmune myasthenia gravis
dsRNA: double-stranded RNA
IFNAR: IFN-I receptor
IRF: interferon regulatory factor
MDA5: melanoma differentiation-associated gene 5
MG: Myasthenia Gravis
PKR: protein kinase R
Poly(I:C): polyinosinic-polycytidylic acid
RIG-I: retinoic acid-inducible gene I
TLR: toll-like receptor
PLP: proteolipid protein

Number of characters in the title: 78

Number of characters in the running head: 47

Number of words in the abstract: 250

Number of words in the manuscript: 3140

Number of references: 41

Number of figures: 6 (no color figure)

ABSTRACT

Objective: Myasthenia Gravis (MG) is an autoimmune disease mediated mainly by anti-acetylcholine receptor (AChR) antibodies. The thymus plays a primary role in MG pathogenesis. As we recently showed an inflammatory and anti-viral signature in MG thymuses, we investigated if pathogen-sensing molecules could contribute to a specific anti-AChR response.

Methods: We studied the effects of diverse toll-like receptor agonists on the expression of α -AChR and various tissue-specific antigens (TSAs) in human TEC cultures. As Poly(I:C), that mimics dsRNA, stimulated specifically α -AChR expression, the signaling pathways involved were investigated in detail. In parallel, we analyzed the expression of dsRNA-signaling components in the thymus of MG patients and the relevance of our data was further investigated *in vivo* after Poly(I:C) injections in mice.

Results: We demonstrate that dsRNA signaling induced by Poly(I:C), specifically triggers the overexpression of α -AChR in human thymic epithelial cells and not of other TSAs. This induction is mediated through toll-like receptor 3 (TLR3) and protein kinase R (PKR), and by the release of interferon (IFN)- β . Interestingly, human MG thymuses as well, display an overexpression of TLR3 and PKR, and overexpress IFN- β .

In addition, Poly(I:C) injections in wild-type mice, but not in IFN-I receptor knockout mice, specifically increase thymic expression of α -AChR and, at later time points, induce an anti-AChR autoimmune response characterized by a significant production of serum anti-AChR antibodies and a specific proliferation of B cells.

Interpretation: Since anti-AChR antibodies are highly specific for MG and are pathogenic, dsRNA signaling activation could contribute to the etiology of MG.

INTRODUCTION

Why do only some people develop autoimmunity? Numerous studies have shown that a genetic background, especially related to the MHC genes, is strongly associated with autoimmunity¹. However, the fact that monozygotic twins do not both systematically develop autoimmune diseases strongly supports the intervention of environmental factors². In this context, pathogen infections have long been suspected³. However, since the onset of symptoms related to an autoimmune disease generally occurs well after a possible triggering infection, it is difficult to link these two events. Cells, and not only cells from the immune system, possess a panel of pathogen-recognition receptors, such as the toll-like receptors (TLR), that recognize specific molecular patterns associated with pathogens, and also endogenous cellular stress signals. The activation of these pathogen-recognition signaling pathways is more and more assumed to play a role in the etiology of autoimmune diseases⁴.

Acquired Myasthenia Gravis (MG) is a neurological autoimmune disease caused by autoantibodies against components of the neuromuscular junction. MG is a prototype organ-specific autoimmune disease with a well-defined antigenic target. Indeed, autoantibodies are mainly directed against the nicotinic acetylcholine receptor (AChR), in particular the α -AChR subunit, which cause the loss of functional AChR and disturb neuromuscular transmission^{5 6}. In MG, if the target organ is the muscle, the effector organ is the thymus which is often characterized by ectopic germinal center development (thymic hyperplasia)⁷. Moreover, the MG thymus includes all the components of the anti-AChR response: AChR⁸, B cells producing anti-AChR antibodies^{9 10}, and anti-AChR autoreactive T cells¹¹.

The thymus is a common target organ for infectious diseases¹² and we recently showed an anti-viral signature in MG thymuses¹³. We then hypothesize that inappropriate activation of pathogen-signal pathways, in genetically predisposed individuals, can lead to autoimmunity. We have investigated in detail how pathogen-sensing molecules could contribute to specific anti-AChR response. Our results show that Poly(I:C) specifically, by mimicking double-stranded RNA

(dsRNA), induces a thymic overexpression of α -AChR and lead to a significant production of anti-AChR antibodies. Since the presence of anti-AChR antibodies is very specific for MG, the production of anti-AChR antibodies in response to Poly(I:C) strongly suggests that dsRNA signaling has a role in the etiology of MG associated with an autoimmune reaction towards AChR.

MATERIELS AND METHODS

All reagents used in this study are detailed in supplementary table S1.

Human thymic samples

Thymic fragments were obtained from MG female patients after thymectomy (16- to 42-year-old, n=29, detailed in Supplementary table S2) or non-MG females (16- to 34-year-old, n=9) and infants (under 3-year-old) undergoing cardiovascular surgery at the Marie Lannelongue hospital (Le Plessis-Robinson, France). All the studies on thymuses and blood samples were approved by the local Ethics Committee (CCP, Ile de France-Paris 7, France - agreement N°C09-36).

Human thymic epithelial (TEC) and myoid cell cultures

TECs were cultured from thymic explants as previously described ¹⁴, a method that favors the development of medullary TECs ¹⁵. 7-day-old cultures were trypsinized and seeded (1.4×10^5 cells/cm²) in RPMI-5% horse serum. After 24 hours, cells were treated with various compounds in RPMI-0.5% horse serum.

MITC (Myoid immortalized thymic cells) were cultured as previously described ¹⁶, seeded at 7000 cells/cm² and treated as for TECs.

Analysis of TECs by flow cytometry

TECs were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) for TLR3 and α -AChR with an antibody against the extracellular domain (Table S1).

Reverse transcription and real-time PCR

Total RNA extracted from cells or tissues was reverse transcribed and analyzed by real-time PCR on the LightCycler apparatus as previously described ¹⁶ using primers listed in supplementary table S3. PCR results were normalized to *GAPDH* for cell cultures or to 28S for thymic tissues.

In vivo experiments

Five-week-old female C57/BL6 (Charles River, France) or IFN-I receptor knockout (IFN- α/β -R under C57/BL6 background (CNRS-Transgônèse, Orleans, France)) mice were injected (i.p.) with 200 μ g of poly(I:C) or physiological water.

For short-term experiments, mice were injected three times every other day and sacrificed at day 6. Their thymus was isolated, weighted and processed for RNA extraction. For long-term experiments, mice were injected every three days and serum was prepared from retro-orbital blood at 2, 4 and 6 weeks. Mice were sacrificed after 6 weeks. Cells from the thymus and accessory axillary and mandibular lymph nodes were isolated by Dounce homogenization for proliferation assays. A thymic fragment was also processed for RNA analysis. Animals were handled according to the animal care and use of laboratory animal guidelines of the French Ministry of Agriculture.

Cell proliferation assays

Mouse thymic and lymph node cells were labeled with 5 μ M CFSE and seeded for three days in 96-well plates at 1.10^6 cells/well in 100 μ l of B-cell medium (X-VIVO15 medium, 10 μ M of β -mercaptoethanol, 2.5 μ g/ml of anti-CD40 and 5 μ g/ml of anti-IgM antibodies) supplemented or not with 2 μ g/ml of purified AChR from the electric organ of torpedo fish (T-AChR), 10 μ g/ml AChR peptide (129-145) or 10 μ g/ml of HA peptide (126-138). After three days, cells were analyzed by flow cytometry with anti CD22, CD4 and CD8 antibodies (Table S1).

Measurement of serum anti-mouse AChR antibody titers

Anti-mouse AChR serum antibodies were tested by radioimmunoassays (RIA) in a blind test, as previously described¹⁷ with the following modifications: 50 μ l of crude extract of denervated mouse muscle were labeled with an excess of 125 I- α -bungarotoxin and incubated with 10 μ l of mouse serum at 4°C overnight. The resulting complexes were precipitated by the addition of 100 μ l of goat anti-mouse Ig, after 4 hours of incubation, and centrifugation at 15,000 x g for 5 minutes. The pellets were washed three times in PBS with 0.5% Triton X-100 and measured in a gamma counter. Titers were corrected for the background of normal mouse serum. The antibody titer was

measured as nanomoles of ^{125}I - α -BT binding sites/L and normalized on the concentration of IgG (g/L).

ELISA

The levels of IFN- β and IFN- α (all subtypes) were analyzed with ELISA kits (Table S1) on 100 μl (1.5 mg/ml) of thymic extract prepared as previously described ¹⁸.

For IgG ELISAs on mouse serum, plates were coated overnight at 4°C with anti-mouse IgGs (1 $\mu\text{g/ml}$). Mouse serum (1/100,000) or standards were incubated for 90 min at room temperature and, subsequently, 1/10,000 of biotinylated anti-mouse IgGs and streptavidin-horseradish peroxidase were added. Tetramethylbenzidine was used for color development. Mouse anti-proteolipid protein (PLP) IgGs were measured in serum diluted 1/100 using an ELISA kit (Table S1).

Statistical analyses

In the text or in bar graphs, results are expressed as means of different experiments or samples, or means of duplicate values when a representative experiment is displayed. Error bars represent SEM. For 2-by-2 comparisons, non-parametric Mann-Whithney tests were applied.

RESULTS

Poly(I:C) induces α -AChR expression specifically in TECs

Since an anti-viral signature was observed in MG thymuses¹³, we wondered whether TLR signaling pathways could modulate α -AChR expression in the thymus. We investigated the effects of TLR agonists on α -AChR expression in human TECs and myoid cells¹⁹, a rare cell population with characteristics similar to skeletal muscle cells²⁰. We observed that among all TLR agonists used in the study, only Poly(I:C) triggered a strong and significant expression of α -AChR mRNA in TECs (Fig 1A). This effect was maximum at 50-100 μ g/ml and induced a 14.7 ± 2.2 (n=12) fold increase in α -AChR mRNA after 24 hours. Kinetic studies showed that α -AChR expression peaked at around 9-12 hours, decreased slightly thereafter and remained elevated up to 72 hours (supplementary Figs S1A-B). Analyzing the effect of TLR agonists on thymic myoid cells, we did not observe any increased expression of α -AChR even with Poly(I:C), suggesting that either α -AChR expression is differentially controlled or that dsRNA signaling is altered in myoid cells compared to TECs (Fig 1B).

Using an antibody against the extracellular domain of α -AChR, we also detected a small but significant increased expression of AChR at the protein level in TECs treated with Poly(I:C) (Fig 1C).

Altogether, these results demonstrate that Poly(I:C), compared to other TLR agonists, selectively triggers the overexpression of α -AChR in TECs.

Poly(I:C) specifically induces α -AChR and not other tissue-specific antigens (TSAs)

The next step was to determine if Poly(I:C) could also affect the expression of other TSAs involved in autoimmune diseases (Supplementary Table S4). We observed that Poly(I:C) increased only α -AChR expression and not other AChR subunits or TSAs (Figs 2A-B), even if Poly(I:C) can induce the expression of the autoimmune regulator AIRE known to control the expression of numerous TSAs (Fig 2C). Altogether, this suggests that α -AChR induction by Poly(I:C) is AIRE-independent.

Poly(I:C) triggers α -AChR expression through TLR3 and PKR signaling pathways

By mimicking dsRNA, Poly(I:C) can interact with TLR3, protein kinase R (PKR)²¹ and RNA helicases (MAD5 and RIG-I)²². We observed that TECs express these dsRNA-sensing molecules and that their expression was up-regulated in response to Poly(I:C) (Fig 3A), suggesting that TECs could play a direct role in innate immunity.

We showed by FACS that TLR3 was located in the intracellular compartment but also on the surface of TECs (Fig 3B). TEC pretreatment with a TLR3 blocking antibody did not decrease the effect of Poly(I:C) on α -AChR expression indicating that the Poly(I:C) effect was not mediated via TLR3 on TEC's surface (Fig 3C). However, TEC pretreatment with a global TLR3 inhibitor (bafilomycin) or a PKR inhibitor (2-aminopurin) strongly inhibited Poly(I:C)'s effect on α -AChR expression (Fig 3D). Moreover, by simultaneously blocking TLR3 and PKR signaling pathways, we almost completely inhibited the induction of α -AChR expression by Poly(I:C) (Fig 3D). Altogether, these results suggest that Poly(I:C) acts intracellularly in TECs through TLR3 and PKR.

Poly(I:C) induces α -AChR expression in TECs cultures via IFN- β release

Since dsRNA triggers an anti-viral response by rapidly inducing IFN-I expression, we studied IFN- α and IFN- β expression in TECs. At the mRNA level, Poly(I:C) strongly induced IFN- β mRNA expression with two peaks of expression around 3 and 12 hours. Thereafter, IFN- β expression decreased but remained elevated (28.2 ± 9.3 fold increase at 24 hours; $n=6$) (Fig 4A). IFN- $\alpha 2$ (an IFN- α subtype with potent antiviral activity) mRNA expression slightly increased 3-6 hours after Poly(I:C) treatment and decreased to the control level, thereafter (Fig 4A). These results prompted us to investigate whether the effect of Poly(I:C) on α -AChR expression was mediated by IFN-I. TEC pretreatment with an IFN-I receptor or with IFN- β blocking antibodies strongly inhibited about 75% of the effect of Poly(I:C) on α -AChR mRNA expression (Fig 4B).

These observations indicate that Poly(I:C) induces α -AChR expression in TECs through the release of IFN-I, mainly IFN- β .

Molecules involved in dsRNA signaling pathway are overexpressed in the thymus of MG patients

We then investigated the expression of dsRNA-signaling components in the thymus of MG patients, and we observed the up-regulation of dsRNA sensing molecules, such as TLR3 and PKR but not RIG1 and MDA-5 (Fig 5A), compared to non-MG adults. We also showed the overexpression in the MG thymus of interferon regulatory factors, IRF7 and IRF5 but not IRF3 (Fig 5B), that are transcription factors involved in dsRNA signaling triggering a cascade of antiviral responses²³.

In a previous analysis of the thymic transcriptome, we already demonstrated the up-regulation of IFN-I-induced genes^{13 24}. Here, by ELISA, we showed increased levels of IFN- β but not of IFN- α in the thymus of MG patients (Figs 5C-D).

The up-regulation of dsRNA-signaling molecules together with the increased expression of IFN- β shows that MG thymuses possess the hallmarks of dsRNA signaling activation.

Injection of Poly(I:C) in mice triggers thymic α -AChR expression and the production of anti-AChR antibodies in the serum

To investigate the relevance of our data in an *in vivo* model, C57Bl6 mice were regularly *i.p.* injected with Poly(I:C). Mice were sacrificed after one week and, as previously observed^{25 26}, Poly(I:C) injections caused thymic involution (data not shown). Most importantly, we observed a significant increase expression of α -AChR but not of β -AChR mRNA in the thymus of Poly(I:C)-injected mice (Figs 6A-B). Poly(I:C) also triggered an increased thymic expression of IFN- β and IFN- α 2 mRNA (Figs 6C-D). Interestingly, induction of α -AChR expression in Poly(I:C)-injected mice was not observed in IFN-I receptor (IFNAR) knockout mice (Fig 6E). These results demonstrate that Poly(I:C), via an IFN-I signaling pathway, can increase the expression α -AChR in the thymus of mice.

We also investigated the effects of Poly(I:C), in long-term experiments, on serum levels of anti-AChR antibodies in comparison to IgGs. We showed a global increase of IgGs after 2 and 4 weeks

but these differences disappeared after 6 weeks (Fig 6F). Using RIA assays, we also observed at 2 and 4 weeks a significant production of serum anti-AChR antibodies that followed those observed for all IgGs. However, after 6 weeks, anti-AChR titers were significantly detected in Poly(I:C)-injected mice even after the normalization of IgG levels (Fig 6G). In parallel, we measured anti-PLP antibodies but did not observe differences between control and Poly(I:C) mice after normalization of IgG levels (Fig 6H).

Proliferation of purified thymic cells and lymph node cells were analyzed in the presence of T-AChR, AChR peptide or HA peptide as antigens. For thymic cells, we observed a non-selective increased proliferation of CD4⁺ T, CD8⁺ T and also B cells from Poly(I:C)-injected mice compared to control mice, regardless of the culture conditions (data not shown). For lymph node cells, we also observed an increased proliferation rate of B cells from Poly(I:C)-injected mice. However, the proliferation rates were higher for cells from Poly(I:C)-injected mice when they were cultured with AChR peptide or T-AChR compared to HA peptide or control medium (Fig 6I) leading to higher percentages of B cells in cultures after 3 days (Fig 6J).

Altogether, these results suggest that Poly(I:C) injections can induce *in vivo* an autoimmune response against AChR.

DISCUSSION

In early-onset MG with anti-AChR antibodies, the thymus is clearly involved in the pathogenesis⁷. Here, we observed the up-regulation of dsRNA-sensing molecules and downstream signaling molecules in the thymus of MG patients. In parallel, we demonstrated that dsRNA signaling activated by Poly(I:C) can induce thymic and peripheral changes leading to an autoimmune response against AChR. Below, we analyze in detail how increased signaling through dsRNA-sensing molecules could be related to MG etiology.

The effect of dsRNA on the thymus has already been investigated in Poly(I:C)-injected mice. It is clear from these studies and ours, that Poly(I:C) causes thymic involution that could disrupt T-cell development^{25 26}. Here, we demonstrated that Poly(I:C) specifically induces thymic overexpression of α -AChR in human TECs, and in the thymus of mice. We have shown that the Poly(I:C) effect is specific for the α -AChR subunit compared to other TSAs. The level of TSA expression in TECs seems to be a key factor for tolerization^{27 28}. According to this postulate, a higher expression of α -AChR in TECs should favor tolerization for this autoantigen. However, our results indicate that, even if Poly(I:C) stimulate AIRE expression, its effect on α -AChR was not AIRE dependent. It has been described that AIRE expression induces apoptosis of mTECs which could favors cross-presentation of TSAs by dendritic cells after phagocytosis of apoptotic cells²⁹. Consequently, the induction of AIRE expression by Poly(I:C) in our TEC cultures could account for the slight increase in apoptosis that we observed but not for TSA regulation (Supplementary Fig S2). We previously demonstrated a strong inflammatory signature in the thymus of MG patients^{13 24}. We can thus hypothesize that a high expression of α -AChR, in an inflammatory environment, together with increased cell death could result in the release of α -AChR fragments and their capture by thymic dendritic cells, thereby triggering autosensitization against AChR characteristic of the MG thymus. The fact that Poly(I:C) induces specifically AChR overexpression and no other TSA could explain why some patients could develop MG and not another autoimmune disease.

In our *in vivo* experiments, we showed that Poly(I:C) injections produce serum anti-AChR antibodies and induce lymph node B cells to proliferate more strongly in the presence of T-AChR or AChR peptide. Thus, we demonstrated that the injection of a non-specific molecule, mimicking dsRNA, can lead to a specific autoimmune reaction against AChR. Since anti-AChR antibodies are specific for MG, this suggests that increased dsRNA signaling could be involved in the human MG etiology. In the human pathology, the thymus is often characterized by ectopic germinal center development (thymic hyperplasia). However, in Poly(I:C)-injected mice, we did not observe more B cells in the thymus or an increased proliferation rate of thymic B cells cultured with T-AChR or AChR peptide. This absence of B-cell signature in the mouse thymus need to be further investigated.

When investigating the molecular mechanisms leading to α -AChR expression by TECs, we demonstrated that the Poly(I:C) effect is mediated through TLR3 and PKR, and subsequently the release of IFN- β which triggers α -AChR expression in TECs. Most importantly, the thymus of MG patients shows the hallmarks of dsRNA activation with the overexpression of TLR3, PKR, and of transcription factors of the IRF family and of IFN- β . The overexpression of dsRNA-sensing molecules has also been observed at the inflammatory sites of other autoimmune diseases^{30 31 32}. The fact that dsRNA signaling molecules and INF- β are still up-regulated in MG patients at the time of thymectomy attests of a chronic inflammatory state in the MG thymus.

How to relate dsRNA signaling activation and MG development? As dsRNA is either the genetic material of certain viruses and/or is produced during the replication cycle of many viruses, the first hypothesis is that MG could develop after viral infection. The thymus is a common target organ for infectious diseases¹². Bernasconi et al. observed increased TLR4 expression in the hyperplastic thymus of MG patients, linking innate immunity and MG³³. In addition, this research group observed that poliovirus-infected macrophages are present in the thymus of some MG patients³⁴ and dysregulated Epstein-Barr virus (EBV) infection in MG thymus³⁵. All these data support the

idea that MG could be the outcome of a viral infection targeting the thymus. In addition, polymorphisms on genes involved in the dsRNA or the IFN-I signaling pathways could also influence susceptibility to viral infections leading to MG predisposition ³⁶. For example, polymorphisms in the IRF5 promoter have been associated with lupus and Sjogren's syndrome ^{37 38}. However, another possibility is that endogenous ligands could be involved in MG onset independent of any primal viral infection. Indeed, TLR3 can be activated by small RNA ³⁹, or by mRNA released from necrotic cells ⁴⁰.

In this study, we demonstrated that dsRNA signaling activation can induce an autoimmune response against AChR. dsRNA could play a central role in MG etiology by inducing specifically α -AChR expression in thymic epithelial cells through the release of IFN- β . These modifications could lead to the emergence of autoreactive T cells against AChR and to the development of an autoantibody response against AChR which is characteristic of MG. Altogether, these results have substantial implications for the understanding of autoimmune pathogenesis that could emerge subsequently to pathogen infections or the used of TLR agonists as vaccine adjuvants ⁴¹.

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Figure Legends

Figure 1: Poly(I:C) induced α -AChR expression specifically in TECs

(A) Effect of TLR agonists on α -AChR mRNA expression in TECs after 24 hours in arbitrary unit (AU) (Pam₃CSK₄ 100 ng/ml for TLR1/2; Poly(I:C) 100 μ g/ml for TLR3; LPS 1 μ g/ml for TLR4; flagellin 100 ng/ml for TLR5; MALP-2 100 ng/ml for TLR6/2; imiquimod 5 μ g/ml for TLR7; R-848 5 μ g/ml for TLR7/8 5 μ g/ml and GpG ODN 2216 for TLR9).

(B) Effect of 100 μ g/ml of Poly(I:C) on α -AChR mRNA expression in MITCs after 24 hours. A representative experiment out of two is shown.

(C) FACS analysis of Poly(I:C) effect (100 μ g/ml) on α -AChR expression on TEC surface after 24 or 48 hours of treatment. FACS graphs show a representative experiment for untreated (control) and Poly(I:C)-treated cells with the dashed line corresponding to the IgG2a control. The dot graph represents the FACS analyses of α -AChR expression (relative geomean of fluorescence) obtained with TECs from five different donors. Dashed line corresponds to α -AChR expression level in control untreated cells normalized to 100 in each experiment.

p-values were assessed by the analysis of variance followed by Bonferonni post tests (a) or by the Mann-Whitney test (B-C), and only p-values < 0.05 are indicated.

Figure 2: Poly(I:C) induced specifically α -AChR expression compared to other TSAs

(A) Effect of Poly(I:C) on α - (black bars), β - (white bars) and ε - (grey bars) AChR mRNA expression in TECs. (B) Effects of Poly(I:C) 100 μ g/ml on mRNA expression of various TSAs listed in table S1 (Supplementary Table S1). (C) Effect of Poly(I:C) 25 to 100 μ g/ml on AIRE mRNA expression in TECs. (A-C) were normalized to GAPDH. Data on graphs are given in arbitrary unit (AU) and correspond to mean values of experiments with TECs from at least four different donors and each experiment was normalized to 100 for untreated cells. p-values were assessed by the Mann-Whitney test and only p-values < 0.05 are indicated.

Figure 3: Signaling components involved in Poly(I:C) effects on α -AChR mRNA expression in TECs.

(A) The effect of 100 μ g/ml of Poly(I:C) on TLR3, PKR, RIG1 and MDA5 mRNA expression in TECs (AU; arbitrary unit). mRNA expression was analyzed after 24 hours, normalized to GAPDH and for each experiment the mRNA level of control untreated cells was fixed at 100. The graph represents the mean of four experiments with TECs from different donors. p-values were assessed by the Mann-Whitney test and p-values < 0.05 are indicated.

(B) FACS analysis of TLR3 expression in TECs. FACS graphs show a representative experiment for TLR3 staining on TEC surface (upper graph) or in the intracellular compartment after permeabilization (lower graph).

(C) α -AChR mRNA expression in TECs pretreated with a blocking TLR3 antibody before Poly(I:C) 100 μ g/ml treatment for 24 hours. α -AChR mRNA expression was normalized to GAPDH. One representative experiment out of three is shown.

(D) α -AChR mRNA expression in TECs pretreated with bafilomycin (Baf) and 2-aminopurin (2-AP), TLR3 and PKR inhibitors respectively, before treating the cells with Poly(I:C) 100 μ g/ml for 24 hours. α -AChR mRNA expression was normalized to GAPDH. One representative experiment out of four is shown.

Figure 4: Poly(I:C) induced α -AChR mRNA expression in TECs via the release of IFN-I

(A) Kinetic effect of Poly(I:C) 100 μ g/ml on IFN- β and IFN- α mRNA expression in TECs. IFN mRNA expression was normalized to GAPDH and the results are given as ratios of mRNA expression in treated versus untreated cells. One representative experiment out of three is shown.

(B) α -AChR mRNA expression in TECs pretreated with blocking antibodies for IFN-I receptor (IFNAR) or IFN- β prior to Poly(I:C) 100 μ g/ml treatment for 24 hours. α -AChR mRNA expression was normalized to GAPDH and for each experiment the mRNA levels of Poly(I:C)-treated cell were fixed at 100 (AU; arbitrary unit). Graph bar represents the mean of four experiments with

TECs from different donors. p-values were assessed by the Mann-Whitney test and p-values < 0.05 are indicated.

Figure 5: Increased expression of dsRNA signaling components in the thymus of MG patients.

(A-B) mRNA expression level for TLR3, PKR, RIG1, MDA5 (A) and IRF3, IRF7 and IRF5 (B) in the thymus of non-MG adult patients (Ad, n=7) and AChR⁺ MG patients (MG, n=15). mRNA expression was normalized to 28S RNA (AU; arbitrary unit). (C-D) ELISAs for IFN- β (C) and all IFN- α isoforms (D) measuring IFN levels in the thymus of non-MG adult patients (Ad, n=8) and AChR⁺ MG patients (MG, n=18). (A-D) p-values were assessed by the Mann-Whitney test and p-values < 0.05 are indicated.

Figure 6: Poly(I:C) injections in mice modified thymic gene expression and induced an autoimmune response against AChR in periphery

(A-D) C57Bl6 or (E) IFNAR KO mice were injected (*i.p.*) with 200 μ g of poly(I:C) or physiological water three times every other days and sacrificed at day 6. Thymic analysis of mRNA expression for α -AChR (A, E), β -AChR (B), IFN- β (C) and IFN- α 2 (D) by real-time PCR normalized to 28S RNA expression (AU; arbitrary unit).

(F-J) C57Bl6 mice were injected as described above every three days for 6 weeks. IgG ELISA on serum obtained at 2, 4 and 6 weeks from control (-●-) and Poly(I/C) (-■-) injected mice (F). Anti-AChR antibody titer was measured by RIA as nmoles of ¹²⁵I- α -BT binding sites per liter (G), ELISA for anti-PLP antibodies (H), and these data were normalized on the concentration of total IgG (g/L) in serum prepared from mice after 6 weeks of injections. Mice were sacrificed after 6 weeks and the lymph node purified cells were labeled with CFSE and cultured in B-cell medium supplemented or with purified AChR (T-AChR), α -AChR(129-145) fragment or HA(126-138) peptide. After 3 days, cells were recovered and the B cells were labeled with an anti-CD22 antibody. Shown is the ratio of CFSE fluorescence geomeans for CD22⁺ cells from Poly(I:C) versus control injected mice (I). Percentage of CD22⁺ B cells after 3 days in culture (J). For these

experiments, data are the means of six mice per group. p-values were assessed by the Mann-Whitney test and p-values < 0.05 are indicated.

Figure 1

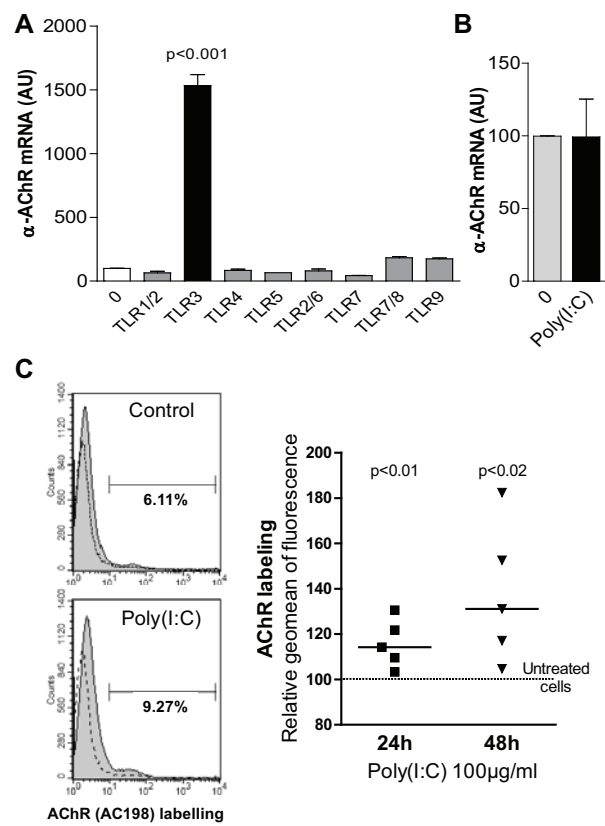


Figure 2

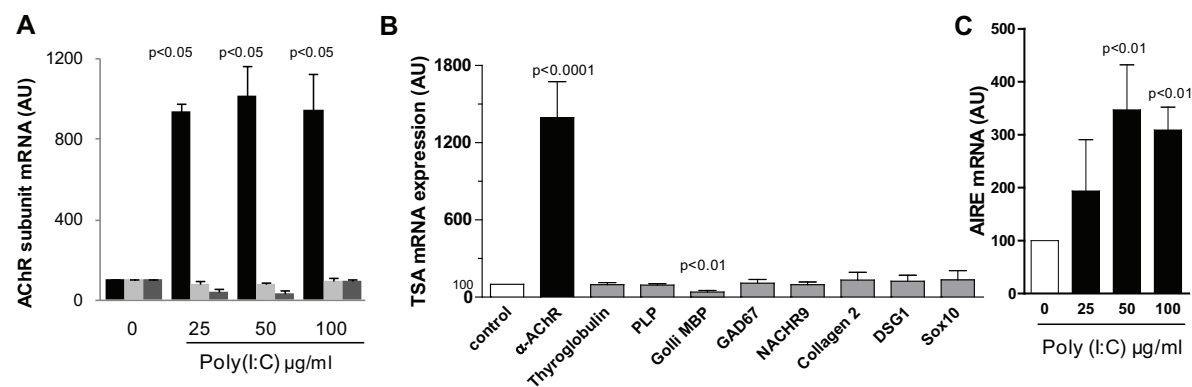


Figure 3

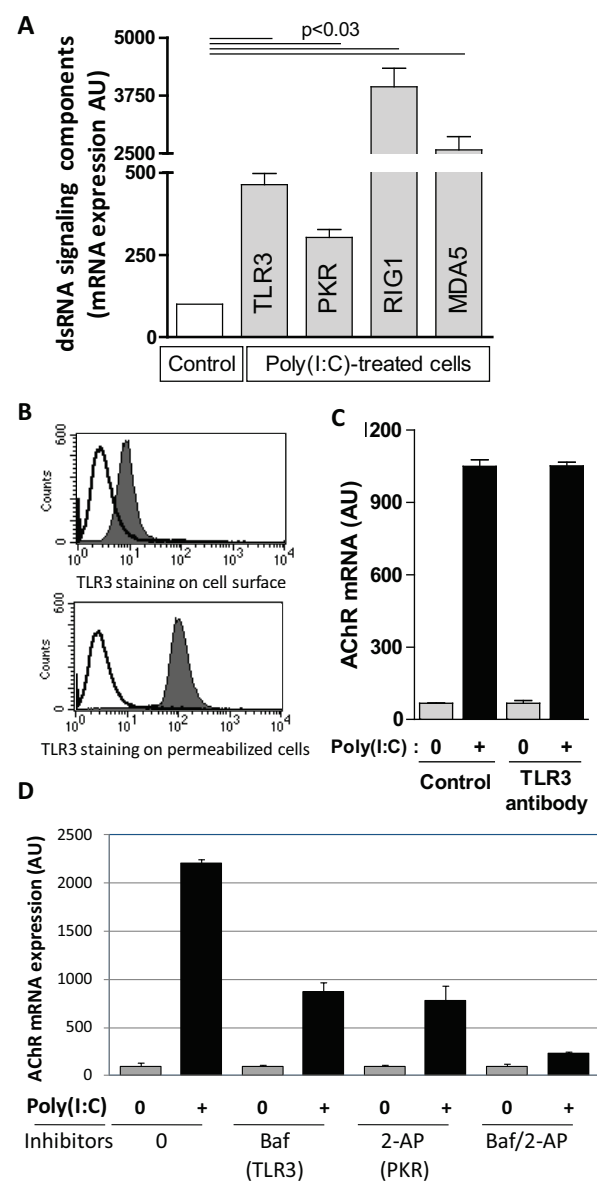


Figure 4

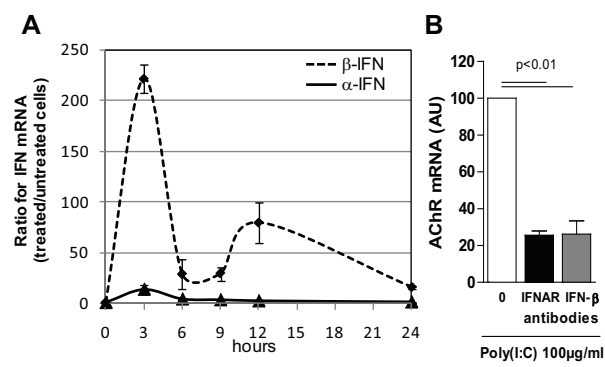


Figure 5

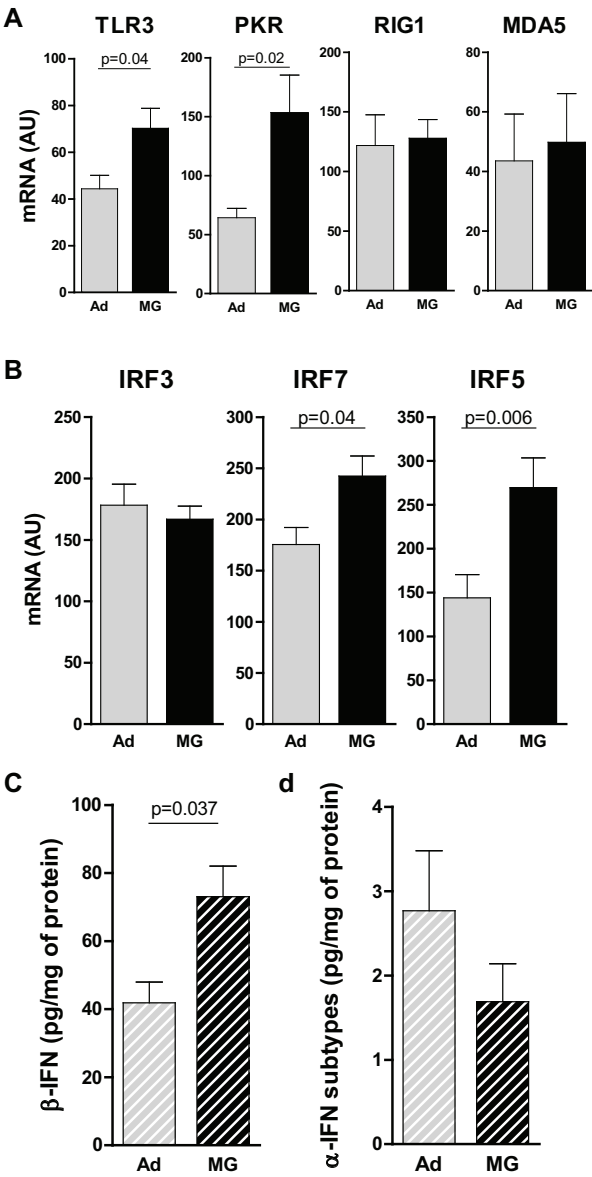


Figure 6

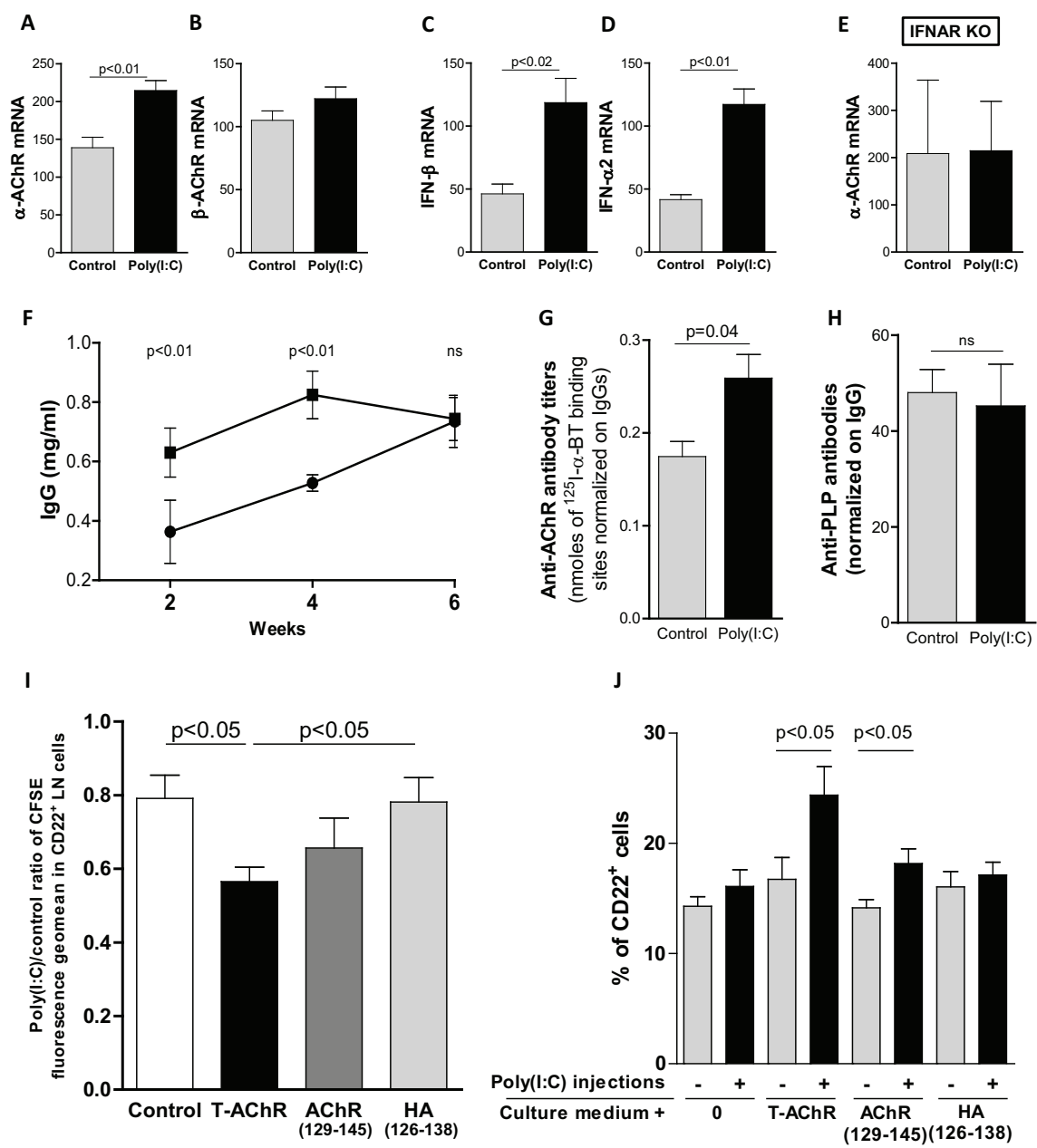


Table S1 : List of the reagents and manufactures

Reagents		
Reagents for cell treatments	Polyinosinic-polycytidylic acid (Poly(I:C)) TLR agonist set	InvivoGen (Toulouse, France) Alexis Biochemical (Enzo Life Sciences, Villeurbanne, France)
Inhibitors	2-Aminopurine (2-AP) Bafilomycin A1 (Baf)	InvivoGen (Toulouse, France) Alexis Biochemical (Enzo Life Sciences, Villeurbanne, France)
Blocking antibodies	Mouse TLR3 antibody Mouse anti-human interferon alpha/beta receptor chain 2 antibody Goat anti-human IFN- β antibody Mouse IgG1 (control) Mouse IgG2a (control) Goat IgG (control)	Abcam (Paris, France) R&D Systems (Lille, France) R&D Systems (Lille, France) Dako (Trappes, France) Dako (Trappes, France) R&D Systems (Lille, France)
Products for TEC and MITC cultures		
Basic cell-culture products	Sera General cell-culture products	Eurobio (Les Ulis, France) Invitrogen (Cergy-Pontoise, France)
Antibodies for TECs analysis by flow cytometry		
Antibodies	Rat anti-human α -AChR antibody (AC198) Rat IgG (control) Goat anti-rat antibody (RPE) Mouse anti-human TLR3 antibody (RPE) Mouse IgG1 (RPE)	Provided by Dr. Tzartos (Hellenic Pasteur institute, Athens, Greece) Becton Dickinson (Le Pont de Claix, France) Becton Dickinson (Le Pont de Claix, France) Abcam (Paris, France) Dako (Trappes, France)
Products for mouse cell cultures and analyses		
Products for B-cell cultures	X-VIVO 15 medium β -mercaptoethanol Hamster anti-mouse CD40 antibody Goat anti-mouse IgM μ , antibody Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Lonza (Levallois-Perret, France) Sigma Aldrich (Lyon, France) Becton Dickinson (Le Pont de Claix, France) Jackson ImmunoResearch Europe (Suffolk, UK) Roche Diagnostics (Meylan, France)
Antibodies for flow cytometry	Rat anti-mouse CD4 antibody (APC) Rat anti-mouse CD8 antibody (PE-Cy5) Mouse anti-mouse CD22 antibody (PE) Rat anti-mouse IgG1 (APC) or (PE-Cy5) or (PE)	Becton Dickinson (Le Pont de Claix, France) Becton Dickinson (Le Pont de Claix, France) Becton Dickinson (Le Pont de Claix, France) Becton Dickinson (Le Pont de Claix, France)
Reagents for cell treatments	Purified AChR from the electric organ of torpedo fish (T-AChR) AChR peptide (fragment 129-145) Influenza hemagglutinin peptide HA (126-138) (HNTNGVTAACSHE)	Provided by Prof. Brenner (Hadassah Medical School, Jerusalem, Israel) Sigma Aldrich (Lyon, France) Provided by Dr. Cohen-Kaminsky (INSERM U999, Le Plessis-Robinson, France)
Reagents for ELISAs		
Human IFN- γ ELISA kits	Human IFN- β ELISA kit Human IFN- α ELISA kit	R&D Systems (Lille, France) R&D Systems (Lille, France)
Mouse IgG ELISA	All antibodies and standard IgGs Streptavidin-horseradish peroxidase (HRP)	Dako (Trappes, France) Beckman Coulter (Villepinte, France)
Mouse PLP ELISA Kit	Anti-proteolipid protein (PLP) IgG quantitative ELISA kit	Eurogentec (Angers, France)

Table S2 : List of AChR positive MG females included in the study

Only AChR positive MG caucasian females, with no other known disease (including thymomas) and only treated with anti-cholinesterase drugs, were included.

Patient	Age (years)	Degree of thymic hyperplasia ⁽¹⁾	Interval (months) Onset - Thymectomy	MGFA ⁽²⁾ score before thymectomy	Anti-cholinesterase drugs	Anti-AChR titer (nmol/L)	PCR ⁽³⁾	ELISA ⁽³⁾
MG1	14	High	24	I	Yes	64.0	X	
MG2	14	High	4	III	Yes	6.6	X	
MG3	16	Low	12	III	Yes	10.3	X	
MG4	18	High	24	II	Yes	33.0	X	
MG5	18	High	36	III	Yes	29.0	X	X
MG6	19	Low	9	IIA	Yes	21.3	X	X
MG7	20	High	8	IVB	Yes	92.7	X	
MG8	20	High	24	II	Yes	1263.0		X
MG9	20	High	4	IV	Yes	3.4		X
MG10	21	High	-	II	Yes	14.8		X
MG11	22	High	2	III	Yes	8.2	X	
MG12	22	Low	9	III	Yes	4.8	X	X
MG13	22	High	2	IIIA	Yes	264.0		X
MG14	23	High	15	IIIB	Yes	105.4	X	
MG15	24	Low	4	III	Yes	1.1	X	X
MG16	25	High	24	III	Yes	23.4		X
MG17	26	High	12	III	Yes	60.0		X
MG18	27	Low	72	III	Yes	26.9		X
MG19	28	High	20	IIA	Yes	123.6	X	
MG20	28	High	48	II	Yes	9.7		X
MG21	29	Low	24	II	Yes	9.2		X
MG22	29	Low	48	III	Yes	15.8		X
MG23	29	High	12	IIA	Yes	179.0		X
MG24	30	Low	93	IIA	Yes	3.4	X	
MG25	31	High	9	I	-	3180.8	X	
MG26	32	Low	36	III	Yes	88.2	X	
MG27	32	High	12	III	Yes	26.7		X
MG28	33	Low	4-5	III	Yes	14.0	X	X
MG29	41	High	72	III	Yes	21.3		X

(1) Degree of thymic hyperplasia: low hyperplasia (with 2 or fewer germinal centers per section) or high hyperplasia (with 3 or more GCs per section)

(2) Myasthenia Gravis Foundation of America (MGFA) Clinical Classification

(3) Samples used for PCR and/or ELISA

(-) Not known

Table S3 : List of primers

Gene	Human primers
α -AChR	AAGCTACTGTGAGATCATCGTCAC (f)
	TGACGAAGTGGTAGGTGATGTCC# (r)
β -AChR	ACAGCTACGACAGCTCGGAG (f)
	CTACCTCATCATCCGCCGCA (r)
AIRE	ATGACACTGCCAGTCACGAG (f)
	AGGAGGTGTCCTTCTCAGCA (r)
GAPDH	CGACCACTTTGTCAAGCTCA (f)
	AGGGGTCTACATGGCAACTG (r)
IFN- α 2	TCCTGCTTGAAGGACAGACA (f)
	TTTCAGCCTTTTGGAACTGG (r)
IFN- β	ACGCCGCATTGACCATCTATG (f)
	CGGAGGTAACCTGTAAGTCTGT (r)
IRF3	ATCGGCTTTTGGGTCTGTTA (f)
	GTCTTGGAGCCGGGTAG (r)
IRF5	TTATTCTGCATCCCCTGGAG (f)
	GCTCTTGTTAAGGGCACAGC (r)
IRF7	TACCATCTACCTGGGCTTCG (f)
	GCTCCATAAGGAAGCACTCG (r)
MDA5	ACCAAATACAGGAGCCATGC (f)
	GCGATTTCCCTTCTTTTGCAG (r)
PKR	ACGCTTTGGGGCTAATTCTT (f)
	TTCTCTGGGCTTTTCTTCCA (r)
RIG1	AGAGCACTTGTGGACGCTTT (f)
	TGCAATGTCAATGCCTTCAT (r)
TLR3	AGCCTTCAACGACTGATGCT (f)
	TTTCCAGAGCCGTGCTAAGT (r)
28S	CGGGTAAACGGCGGGAGTAA (f)
	GGTAGGGACAGTGGGAATCT (r)

Gene	Mouse primers
α -AChR	GTGCTGGGCTCTTTCATCTC (f)
	TTCTGTGCGCGTTCTCATAC (r)
β -AChR	GGACCGTCTTTTTCTGTGGA (f)
	GATTCTTACAAGCGCCAAGC (r)
GAPDH	AACTTTGGCATTGTGGAAGG (f)
	ACACATTGGGGGTAGGAACA (r)
IFN- α 2	TCTGTGCTTTCCTCGTGATG (f)
	TTGAGCCTTCTGGATCTGCT (r)
IFN- β	CCCTATGGAGATGACGGAGA (f)
	CTGTCTGCTGGTGGAGTTCA (r)
28S	CGGGTAAACGGCGGGAGTAA (f)
	GGTAGGGACAGTGGGAATCT (r)

Table S4 : List of AIRE-regulated TSAs involved in autoimmune diseases

Autoantigen	Autoimmune disease	Thymic expression (medullary or cortical TECs)	Regulated by AIRE or altered in APS syndrome
Collagen II	Rheumatoid arthritis	cTECs/mTECs ¹	No/Yes ^{2,3}
Desmoglein 1 (DSG1)	Pemphigus	No ⁴	Yes ⁵
Glutamic Acid Decarboxylase 67 (GAD 67)	Type 1 diabetes	mTECs ¹	No ⁶
Golli-Myelin Basic Protein (MBP)	Multiple sclerosis	cTECs/mTECs ¹	No ⁷
Nicotinic AChR- α (AChR subunits)	Myasthenia Gravis	mTECs ⁸	Yes ⁹
Nicotinic AChR- $\alpha 9$	Pemphigus	?	?
Proteolipid protein (PLP)	Multiple sclerosis	cTECs/mTECs ¹	No/Yes ^{10,7}
SOX-10	Vitiligo	?	Yes ¹¹
Thyroglobulin	Hashimoto's thyroiditis	mTECs ¹	Yes ¹²

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Figure S1

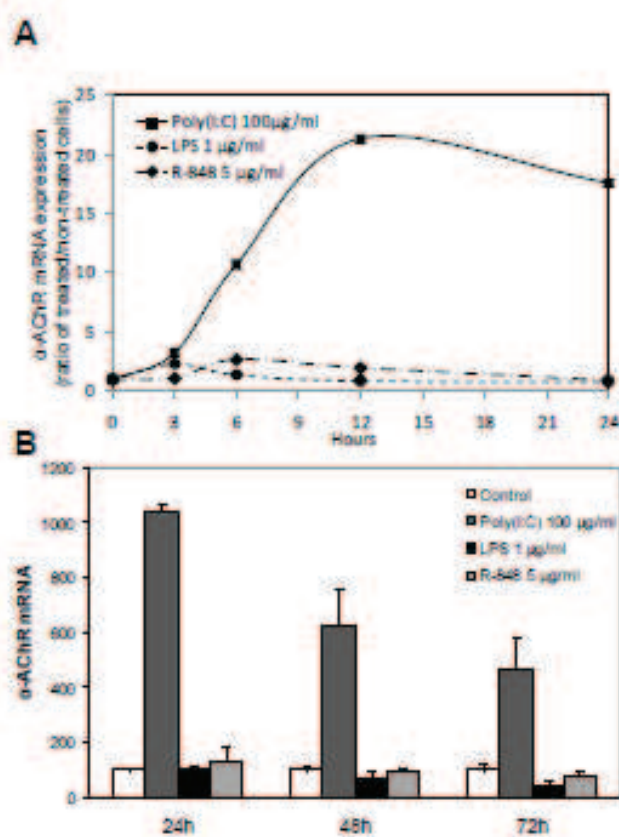


Figure S1: Poly(I:C) induced α -AChR expression in TECs

Kinetic analyses of the effects of Poly(I:C) 100 μ g/ml, LPS 1 μ g/ml and R-848 5 μ g/ml on α -AChR mRNA expression by TECs. (A) Kinetic analyses over 24 hours. α -AChR mRNA expression was normalized on GAPDH and results has been given as ratios of mRNA expression in treated versus untreated cells; Mean of 4 experiments for Poly(I:C) and 2 experiments for LPS and R-848.

(B) Kinetic analyses over 72 hours. α -AChR mRNA expression was normalized on GAPDH and for each time point normalized on control values fixed at 100. Representative experiment out of 2. Error bar for duplicate PCR values.

Figure S2

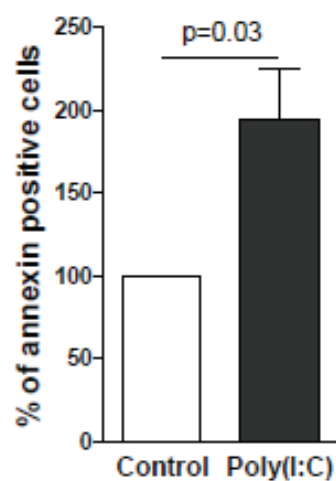


Figure S2: Poly(I:C) increased apoptosis of TECs.

TECs were labeled with annexin-V-FITC and analyzed by flow cytometry. Bar chart corresponds to the percentage of annexin-V-FITC labeled cells. Mean values \pm SEM of four experiments with TECs from different donors and each experiment was normalized to 100 for untreated cells. p-values was assessed by the Mann-Whitney test.

ARTICLE 4

Thymic remodeling associated with hyperplasia in myasthenia gravis

OBJECTIVE

This manuscript is a review about the structural and cellular changes associated with thymic hyperplasia, whose main characteristics include the development of ectopic germinal centers and the development of high endothelial venules and lymphoangiogenesis.

We summarized recent data concerning the thymic changes in MG patients with focus on cellular modifications, inflammatory signature and the role chemokines.

Thymic remodeling associated with hyperplasia in myasthenia gravis

ROZEN LE PANSE^{1,3}, JACKY BISMUTH^{1,3}, GÉRALDINE CIZERON-CLAIRAC^{1,3},
JULIA MIRIAM WEISS^{1,3}, PERRINE CUF^{1,3}, PHILIPPE DARTEVELLE²,
NICOLE KERLERO DE ROSBO^{1,3}, & SONIA BERRIH-AKNIN^{1,3}

¹CNRS UMR 8162, 92350 Le Plessis-Robinson, France, ²Hôpital Marie Lannelongue, 92350 Le Plessis-Robinson, France, and ³Université Paris-Sud, 91405 Orsay, France

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Abstract

Acquired myasthenia gravis (MG), a neurological autoimmune disease, is caused by autoantibodies against components of the neuromuscular junction that lead to disabling muscle fatigability. The thymus is clearly involved in the pathogenesis of early-onset MG with anti-acetylcholine receptor antibodies, and thymic hyperplasia of lympho-proliferative origin is a hallmark of the disease. In this review, we describe the structural and cellular changes associated with thymic hyperplasia, its main characteristics being the development of ectopic germinal centers (GCs) associated with active neoangiogenic processes, such as development of high endothelial venules and lymphangiogenesis. What triggers such thymic abnormalities in MG is not yet clear. A thymic transcriptome analysis has demonstrated a strong inflammatory signature in MG that could orchestrate the development of thymic hyperplasia. In this context, thymic epithelial cells (TECs) seem to play a central role, either by contributing or responding to the inflammatory environment and up-regulating the autoimmune response. In particular, MG TECs clearly overexpress various cytokines, among which chemokines play a crucial role in the recruitment of peripheral lymphocytes to the thymus via the newly expanded vessel network, thereby leading to the development of ectopic GCs. Clearly, a better understanding of major events that lead to thymic hyperplasia will help optimize strategies toward more specific therapy for MG.

Keywords: *Myasthenia, thymus, germinal centers, chemokines, inflammation*

Abbreviations: AChR, acetylcholine receptor; GC, germinal center; HEV, high endothelial venule; IFN, interferon; MG, myasthenia gravis; MHC, self-major histocompatibility complex; MuSK, muscle specific tyrosine kinase; SLO, secondary lymphoid organ; SN, seronegative for AChR antibodies; SP, seropositive for AChR antibodies; TEC, thymic epithelial cell

Introduction

Acquired myasthenia gravis (MG) is a neurological autoimmune disease caused by autoantibodies against components of the neuromuscular junction [1] that lead to disabling fatigability. Most MG patients (about 85%) have anti-acetylcholine receptor (AChR) antibodies [seropositive (SP) MG] [2], and a minor subgroup (<5%) have anti-muscle-specific tyrosine kinase (MuSK) antibodies [3]. There are approximately 10% of patients without known antibodies [seronegative (SN) MG]. However, this distinction is misleading as these patients respond well to plasma exchange and their plasma can transfer the disease to experimental animal models [4]. Recently, Leite et al.

[5] have demonstrated that some SN patients could in fact correspond to SPMG patients with anti-AChR antibody that are not detectable by routine immunoprecipitation. Although the muscle is the target organ in this disease, the thymus is clearly involved in the pathogenesis of SPMG.

The thymus provides a complex environment essential for the generation of the T-cell repertoire. It is composed of various cell types, essentially thymocytes and thymic epithelial cells (TECs), but also fibroblasts, macrophages, dendritic cells (DCs) and myoid cells [6]. Differentiation of T cells occurs while they are progressing through the different thymic compartments. Successful T-cell differentiation depends on the quality and the specificity of T-cell

receptor/antigen-self-major histocompatibility complex (MHC) interactions (positive selection). Medullary TECs, which express a broad panoply of tissue-specific antigens, play a crucial role in central tolerance by controlling negative selection of autoreactive T cells and by participating to the selective induction of regulatory T cells [7,8].

In MG, histological abnormalities are very often found in the thymus, which displays either thymic hyperplasia or thymoma [9]. In SPMG, especially early onset cases, 50–60% of the patients exhibit thymic hyperplasia of lympho-proliferative origin characterized by ectopic germinal center (GC) development [10] (Figure 1(A)). Thymic hyperplasia is especially correlated with the anti-AChR antibody titer (Figure 1(B)), which decreases after thymectomy [11]. The hyperplastic thymus includes all the components of the anti-AChR response: AChR [12], B cells producing anti-AChR antibodies [13,14], and anti-AChR autoreactive T cells [15]. The classical animal models of MG are induced in mice or rats via immunization with purified AChR extracted from the electric organ of Torpedo, emulsified in complete Freund's adjuvant. These models do not present thymic abnormalities [16], indicating that the pathological events at the thymus in human MG do not result from the production of anti-AChR antibodies

in the periphery. All these observations support the role of the thymus in the pathogenesis of MG and thymectomy is often advised for SPMG patients, resulting in slow but effective improvement of symptoms over a few years [17,18]. In contrast, there is little information on the involvement of the thymus in the non-SP form of MG. The thymus of MuSK⁺ patients shows few or no pathological changes and the beneficial effects of thymectomy have not been really proven for this subgroup [18,19]. In SN patients, the clinical characteristics are heterogeneous, probably because this group contains patients with different pathogenic autoantibodies [5]. Histological analyses of the thymus have shown that SN patients can present a few GCs as in SPMG [20,21], and thymectomy can be beneficial for some SNMG patients [19].

To establish the presence and nature of thymic pathology, examination of the tissue must take into account the therapy undergone by the patient, which, as demonstrated for corticosteroid treatment (see later), can modulate thymic pathology [22]. To our knowledge, the effects on thymic pathology of other treatments, such as oral azathioprine, intravenous immunoglobulins, or plasmapheresis, have not been reported. How thymic hyperplasia develops in the thymus of MG patients is not yet clearly defined.

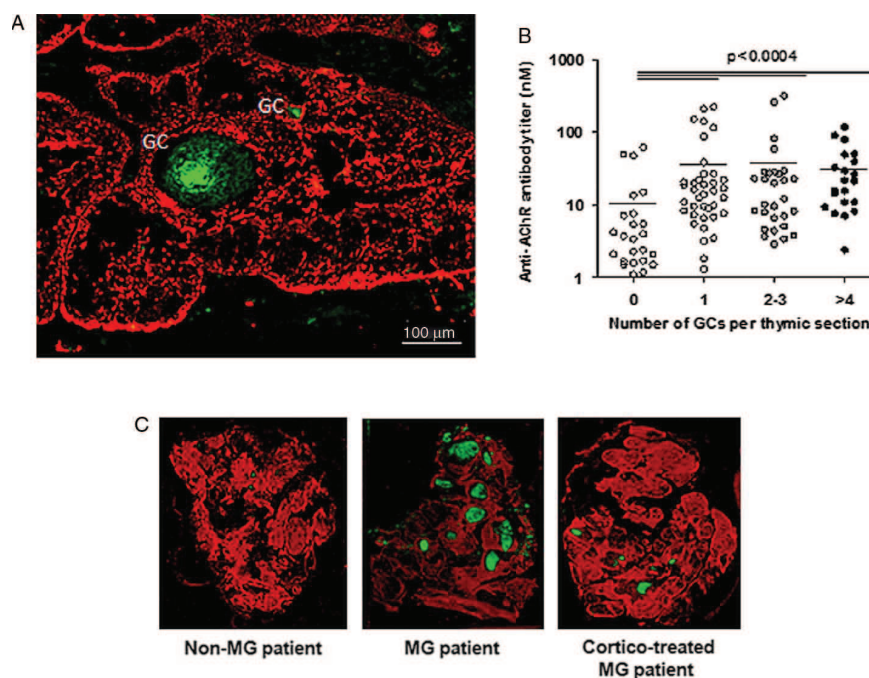


Figure 1. Ectopic GC development in the thymus of MG patients. (A) Immunohistochemical analysis of a thymic section from an MG patient. TECs are labeled in red with anti-keratin antibody and GCs in green with anti-CD21 antibody. (B) Levels of anti-AChR antibody titer according to the mean number of GCs per thymic sections in MG patients (each point represents a different patient). The *p*-values were assessed by the Mann–Whitney test and only *p*-values < 0.05 are indicated. Studies on thymuses and blood samples displayed in this and the following figures were approved by the local ethics committee (CCPPRB, Kremlin-Bicêtre, France, agreement 06-018). (C) Decrease in the number of GCs in thymus of MG patients under corticoid treatment. Immunohistochemical analyses of thymic sections from non-MG patients undergoing cardiovascular surgery, or non-treated MG patients and corticoid-treated MG patients undergoing thymectomy. TECs were labeled in red and GCs in green as above. All thymic fragments were obtained from women aged 22–40 years. Slides were scanned with a 428 Affymetrix scanner as described previously (from Meraouna et al. [22]).

Apparently, a genetic contribution is strongly supported, with the human leukocyte antigen (HLA)-A1-B8-DR3 haplotype being associated with MG characterized by thymic hyperplasia [15], and a possible role for sexual hormones and other gender-related factors is suggested by the higher occurrence of thymic hyperplasia in young female SPMG patients [23].

Here, we review thymic remodeling related to thymic hyperplasia in SPMG, in order to better understand the mechanisms involved in the pathogenesis of MG. For this purpose, we will describe and discuss (1) the structural and cellular changes associated with thymic hyperplasia; (2) the inflammatory signature that might underlie these thymic changes; and (3) the role of chemokines that actively participate in peripheral cell recruitment leading to ectopic GC development.

Structural and cellular changes in hyperplastic MG thymuses

In early onset SPMG, the thymus is the site of profound structural alterations. Bofill et al. [24] observed thymic changes in MG characterized by an unusual arrangement and hypertrophic appearance of medullary TEC areas. However, the main characteristics of MG thymuses are the development of ectopic GCs in perivascular spaces in the medullary region (Figure 1) and active neoangiogenic processes (Figure 2). These changes are accompanied with alterations of the extracellular matrix thymic components. Indeed, a major and unusual connective framework containing collagen types I, III, and IV, as well as laminin and fibronectin, is observed. The basement membrane presents some discontinuity [25], which is in contrast with the continuous line pattern found in the normal thymus. Interestingly, these abnormalities are consistently found in close proximity to GCs [25].

Ectopic GC

As previously stated, the development of ectopic GCs is very often observed in early onset SPMG (Figure 1(A)). These thymic abnormalities correlate with the anti-AChR antibody titer (Figure 1(B)) [10,26], which decreases after thymectomy [11]. A small proportion of B cells are known to be present in normal thymus, increasing with age and even more in hyperplastic thymuses of MG patients [27]. B cells organized within GCs can be occasionally spotted in the thymus of non-MG patients [28,29] and in other autoimmune diseases [30]. Our study of thymic transcriptome in SPMG patients with a high or low thymic hyperplasia and SNMG patients versus non-MG patients has demonstrated that the thymus of all MG patients is characterized by an abnormal increased B-cell population compared

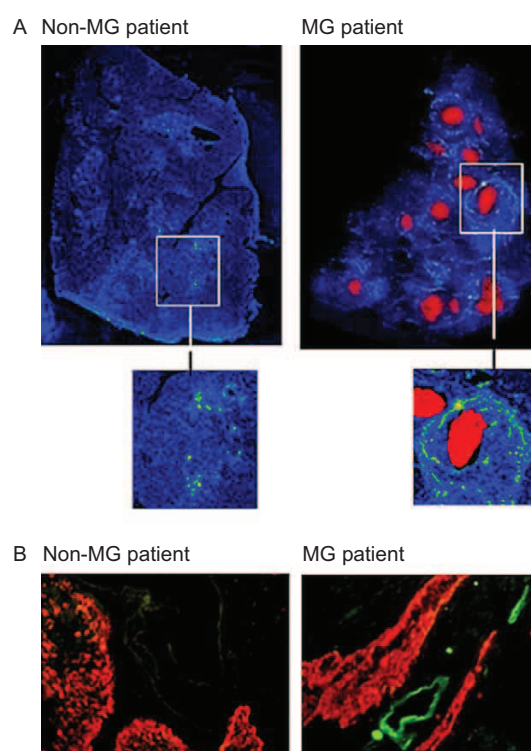


Figure 2. Neovascular processes in the thymus of MG patients. (A) Serial thymic sections from non-MG and MG patients stained with antibodies against CD21 (red), anti-PNAd (green), and anti-keratin (blue) to localize GCs, HEVs, and TECs, respectively. The whole sections were then scanned using a 428 Affymetrix scanner with a resolution of 10 μm , and images were superimposed (bars = 200 μm) (from Berrih-Aknin et al. [39]). (B) Immunohistochemical analyses of thymic sections from non-MG and MG patients. TECs are labeled in red with anti-keratin antibody and CCL21 in green with anti-CD21 antibody (bars = 20 μm) (from Berrih-Aknin et al. [39]).

to non-MG adults. Moreover, even non-hyperplastic thymuses display the characteristics of increased diffuse B-cell infiltrations [31,32]. The thymus of SP patients has been shown to include all the components of the anti-AChR response including B cells producing anti-AChR antibodies, suggesting possible expansion of specific B cells [33]. An overall increased expression of immunoglobulin genes, independent of antigenic specificity, was also observed, demonstrating the large diversity of B cells in MG thymuses [31,34]. This observation is supported by the observation that thymic GCs in MG contain a remarkably heterogeneous population of B cells [35], and by *in situ* hybridization experiments showing the polyclonality of B cells present in individual thymic GCs of MG patients [36].

The beneficial symptomatic effect of glucocorticoid treatment may be related to its biological effect on B cells. Indeed, numerous B-cell markers that are overexpressed in the hyperplastic thymus of MG patients are normalized upon glucocorticoid

treatment. Moreover, histological analysis clearly shows a decreased number of GCs in thymus of glucocorticoid-treated MG patients compared to non-treated MG patients [22] (Figure 1(C)). All these observations support the fact that B-cell infiltrations leading to thymic hyperplasia are central in disease development. Consequently, the development of specific drugs preventing thymic hyperplasia in MG is of major interest and could eventually replace thymectomy or the use of non-specific glucocorticoid treatments.

Neoangiogenesis: "in and out cell trafficking"

Post-capillary venules of lymph-node type in the thymus of MG patients were first described by Soderstrom et al. [37], suggesting that neoangiogenesis occurs in MG thymus. Real-time PCR (RT-PCR) analysis of CD31 revealed a significantly increased expression of this blood-vessel marker in the hyperplastic thymus of MG patients (data not shown), indicative of intense angiogenic processes.

In secondary lymphoid organs (SLOs) and in chronically inflamed tissues, lymphocyte homing is directed through high endothelial venules (HEVs), a specialized endothelium bearing on its luminal surface diverse chemokines and expressing high levels of peripheral node addressin carbohydrate ligands [38]. By immunohistochemistry, only a few HEVs were observed in the thymus of non-MG adults. In contrast, in the thymus of MG patients, increased numbers of HEVs could be seen, which were located around GCs and correlated with the degree of thymic hyperplasia [39] (Figure 2(A)). Such high numbers of HEVs in hyperplastic thymuses suggest that peripheral cells enter MG thymus through these specialized vessels, and investigations are in progress in our laboratory to identify which chemokines are present on these HEVs that could possibly be responsible for such recruitment.

Lymphangiogenesis occurs throughout life in homeostasis and disease. In SLOs, afferent lymphatic vessels selectively convey lymphocytes and antigen-presenting DCs, and efferent lymphatic vessels carry lymphocytes out of lymph nodes ultimately into the major efferent ducts and then back to the circulation [40]. Lymphangiogenesis has been described in lymph nodes after immunization, where it is shown to be dependent on the entry of B cells [41]. Using RT-PCR analysis, we observed an increased expression of lymphatic markers (vascular endothelial growth factor receptor-3 (VEGFR3) and prospero homeobox protein 1 (PROX1)) in hyperplastic thymuses, which also suggests an expansion of the thymic lymphatic system in MG [39]. Moreover, in hyperplastic MG thymuses, we demonstrated the presence of CCL21-positive lymphatic vessels (Figure 2(B)). These vessels also express D6 [39], a decoy receptor for most inflammatory chemokines known to be expressed

on afferent lymphatic vessels of peripheral organs [42]. According to Pearse [43], there is no afferent lymphatic vessel in the normal thymus; thus, in hyperplastic thymuses, CCL21-positive vessels could represent a pathological development of afferent lymphatic vessels, which, in addition to thymic HEVs, could correspond to an entrance of circulating peripheral cells. These thymic afferent lymphatic vessels could also mediate the recruitment of sensitized DCs, as suggested by Nagane et al. [44]. Another hypothesis is that hyperplastic MG thymuses behave similarly to an inflamed peripheral organ where CCL21-positive afferent lymphatic vessels might represent a way out for cells emerging from GCs, such as differentiated B cells or activated T cells, which are CCR7-positive.

In the thymus of MG patients, and in particular in hyperplastic thymuses, the active angiogenic processes characterized by HEV development and lymphangiogenesis support the idea of intense cell trafficking between the thymus and the periphery.

Implication of TECs and myoid cells: changes in AChR expression?

TECs are involved in thymocyte maturation/differentiation processes and the maintenance of tolerance to self. At different levels, TECs could play a central role in MG development and be directly implicated in thymic hyperplasia. First, several studies have demonstrated that TECs overproduce various cytokines in SPMG, such as interleukin-1 (IL-1) [45], IL-2 [46], IL-6 [47,48], and transforming growth factor- β (TGF- β) [49]. All these cytokines could thus alter intrinsic thymocyte differentiation but also participate in the development of Th1 and Th2 responses toward AChR. TECs also overproduce chemokines known to actively recruit peripheral cells (detailed below) and, consequently, participate in thymic hyperplasia.

Second, as TECs play a crucial role in natural regulatory T-cell formation in the thymus, alteration in MG TECs could explain why regulatory T cells are defective in MG [50].

Third, TECs through their expression of tissue-specific antigens also play a key role in self-tolerance education of thymocytes. The level of expression of these tissue-specific antigens in the thymus seems to be a key factor for tolerization. Indeed, Salmon et al. [51], using transgenic mice with a β -galactosidase gene under the control of AChR- α gene promoter, showed that a low level of thymic expression of the transgene does not allow full tolerance and leads to the development of an autoantibody response against β -galactosidase when injected into the transgenic mice. Similarly, in the human insulin-dependent diabetes mellitus, the susceptibility to diabetes is higher in patients with low-insulin thymic expression [52]. TECs are able to express all AChR subunits [12,53].

Table I. Analyses of AChR- α expression in the thymus and in thymic epithelial cell cultures from MG patients compared to non-MG patients.

AChR- α expression MG versus non-MG patients		
Method	Thymic expression	TEC expression
Semiquantitative PCR	Increased expression of P3A+ and P3A- AChR- α isoforms [52]	Increased expression of P3A+ and P3A- AChR- α isoforms [52]
Semiquantitative PCR	No variation in the expression of P3A+ and P3A- AChR- α isoforms [9]	No variation in the expression of P3A+ and P3A- AChR- α isoforms [9]
Microarrays	Decreased expression of AChR- α (GeneID1134; [29])	ND
RT-PCR	No variation in the expression of AChR- α (unpublished data)	No variation in the expression of AChR- α (unpublished data)

TEC even express the two known isoforms of AChR- α (P3A+ and P3A-) [12,54]. Regarding the level of AChR- α expression in the thymus or TECs from MG and non-MG patients, different results have been obtained that are summarized in Table I. So far, these observations cannot help us determine if a decrease in the thymic level of AChR- α expression is a triggering event in MG, especially because thymic investigations are obviously carried out when disease is full blown, and that could be a long time after the triggering event(s).

Myoid cells in the thymus correspond to a rare cell population localized in the medulla and at the cortico-medullary junction [55,56]. They possess the antigenic characteristics of skeletal muscle cells and express all AChR subunits allowing the expression of a functional AChR receptor [53,56]. In our laboratory, we investigated the myoid cell number on thymic sections and observed fewer myoid cells in the thymus of MG compared to non-MG patients (unpublished data). In a more thorough study, Leite et al. observed enormous variability in the numbers of myoid cells for these patient categories, albeit with some overall decrease per mm² of medullary parenchymal tissue [57]. In the thymus of early onset SPMG patients, myoid cells colocalize significantly with GCs [58]. This suggests that damage to or destruction of myoid cells targeted upon an autoimmune attack could result in the release of AChR fragments and their capture by local antigen-presenting cells, such as TECs or DCs, thereby triggering autosensitization against AChR in MG [57].

Inflammatory signature-role of interferons

As for many autoimmune diseases, the triggering events involved in MG are totally unknown. In MG, as the autoimmune sensitization against AChR develops in the thymus, the role of inflammatory cytokines is crucial in MG pathogenesis. Thymic transcriptome analyses underline the inflammatory state of MG thymuses and, in particular, the overexpression of numerous interferon-I (IFN-I)-

and IFN-II-induced genes [59]. Although these results suggest an increased expression of IFNs, such an increase has not been observed in peripheral mononuclear cells [60,61] or thymus [31,46] from MG patients. These data are similar to those in patients with severe lupus where deregulated expression of IFN pathway genes in peripheral blood cells is not associated with increased expression at the protein or mRNA levels in sera or mononuclear cells, respectively [62,63]. The apparent contradiction between a lack of increase in expression of IFNs in patients and signs of IFN activity could be due to the short half-life of IFNs [64] combined with a transient overproduction of IFNs occurring only at the onset of the disease. In MG studies, thymus samples are collected at least several months after disease initiation, when levels of IFNs may have decreased to normal values. A possible increase in IFN levels at the onset of the disease may be sufficient to induce the activation of many other genes whose expression remain elevated over a longer time frame.

Role of IFN-I

The potential implication of IFN-I in MG has long been suggested in various ways but never clearly defined: (1) clinical reports demonstrate the development of MG after IFN- α - or IFN- β -based therapy [65–68]; (2) antibodies against IFN- α are found in some MG patients [69,70]; and (3) thymic transcriptome analysis of different MG patient subgroups has revealed a significantly increased expression of IFN-I-induced genes [32,59].

IFN-I are secreted by various cells as an antiviral defense mechanism and the thymus is a common target organ in infectious diseases [71]. Moreover, viral infections have been associated with the development of several autoimmune diseases [72]. However, since the onset of symptoms related to an autoimmune disease generally occur well after a possible triggering infection, it is difficult to link these two events. Nevertheless, molecular mimicry hypotheses suggest that autoimmune diseases can be caused directly by T- or B-cell responses to a microbial

antigen that cross-reacts with a self-antigen. Thus, some MG patients have antibodies to AChR which shares a cross-reactive epitope with the herpes simplex virus glycoprotein D [73]. Another infection-induced autoimmunity, referred to as bystander effect, occurs secondary to infection. The initial viral infection induces a localized inflammation of the target organ and triggers an auto-antigen sensitization [72]. In this context, our thymic transcriptome analyses demonstrated an overexpression of genes involved in host response in SN patients [31]. Kott et al. [74] also observed an activated IFN system and defective natural killer cell activity for many MG patients, suggesting an occult viral infection. An increasing number of studies suggest that activation of toll-like receptors (TLRs), pattern recognition receptors that recognize molecules on pathogens, could be involved in autoimmunity [75,76]; in this context, an increased TLR4 expression has been observed in the hyperplastic thymus of MG patients, linking innate immunity and MG [77]. Altogether, these data support the idea that MG could develop consecutively to viral infection targeting the thymus.

Role of IFN-II

IFN-II (also named IFN- γ) has been implicated in various autoimmune diseases because of its pro-inflammatory properties, in particular its ability to induce MHC class I and II expression and to promote Th1 differentiation. In MG, IFN-II by itself can trigger functional disruption of the neuromuscular junction [78]. Attempts to induce experimental autoimmune MG (EAMG) in IFN-II or IFN-II-receptor knockout mice demonstrate that IFN-II is required for the generation of a pathogenic anti-AChR humoral immune response and for conferring susceptibility to clinical EAMG to mice [79,80].

In the thymus, an up-regulation of IFN-II-induced genes is observed and the thorough analysis of the effects of IFN-II on AChR subunit expression in TEC and myoid cell cultures has demonstrated that IFN-II is a strong inducer of AChR subunits and, in particular, of AChR- α [59]. In IFN-II knockout mice, the absence of IFN-II significantly reduces the expression of AChR- β mRNA in the thymus, while it does not have any effect on AChR- β expression in the muscle [59]. An increased expression of IFN-II that was shown to affect the generation of regulatory T cells [81] could also play a role in MG pathogenesis.

Consequently, IFN-II, by up-regulating AChR expression in a pro-inflammatory environment, could alter self-tolerance, affect the generation of regulatory T cells, and trigger an autoimmune response toward AChR.

Role of chemokines in B- and T-cell recruitment to the thymus

Chemokines, through their chemotactic, but also their chemorepulsive properties, play a central role in thymopoiesis allowing the recruitment of pro-thymocytes, the migration of thymocytes from the cortex to the medullary region, and their export to the periphery (Figure 3(A)). Chemokines are also crucial for peripheral cell recruitment in SLOs [82]. As the hyperplastic thymus of MG patients presents the major characteristics of SLOs with HEV and GC development, it is likely that thymic chemokine expression profiles differ in MG patients and are involved in thymic hyperplasia (Figure 3(B)). Such a possibility is supported by results of chemotactic analysis showing significantly increased migration of peripheral blood lymphocytes toward extracts of hyperplastic MG thymus [31]. Moreover, our thymic transcriptome study showed that the number of up-regulated chemokines was significantly over-represented in SPMG [31], in particular chemokines likely to be implicated in thymic hyperplasia, such as CXCL13 and CCL21.

CXCL13

The chemokine CXCL13 is selectively chemotactic for B cells and elicits its effects by interacting with the chemokine receptor CXCR5. In SLOs, CXCL13 is known to participate in GC formation [83]. CXCL13 is also found overexpressed at inflammatory sites characterized with ectopic GC development in various autoimmune diseases, such as rheumatoid arthritis [84]. In normal thymus, CXCL13 is barely expressed. However, increased thymic expression of CXCL13 is observed in aged (NZB \times NZW)F1 (BWF1) mice, a murine model for systemic lupus erythematosus. In this murine model, the increased expression of CXCL13 was attributed to mature myeloid DCs infiltrating the thymus [85]. This increased expression favors the recruitment of peripheral B cells in aged mice that are more prone to develop a lupus.

In MG patients, CXCL13 was demonstrated to be overexpressed in the thymus of SP and SNMG patients, more particularly in highly hyperplastic SPMG thymuses. The increased thymic production of CXCL13, which is due to TECs [22], could explain the generalized thymic B-cell infiltration observed in all MG patient subgroups [31]. Saito et al. [86] observed an increased expression of CXCR5, the receptor for CXCL13, on a subset of CD4⁺T cells, called follicular B-helper T cells, in peripheral blood of MG patients. These cells play a pivotal role in antibody production by B cells in SLOs [87]. Altogether, the increased expression of CXCL13 in the thymus of MG patients and of CXCR5 on follicular B-helper T cells could support the development of ectopic GCs in the thymus of MG patients.

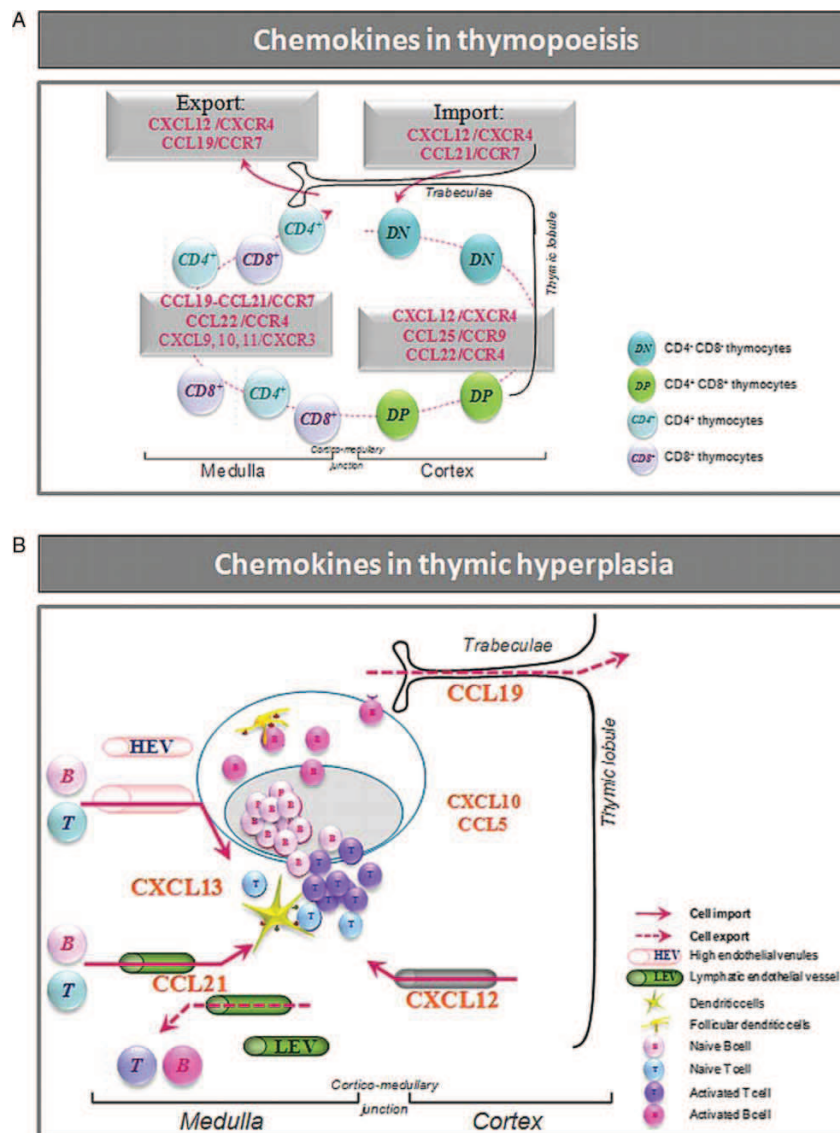


Figure 3. Role of chemokines in thymopoiesis and in thymic hyperplasia. (A) Role of chemokines in thymopoiesis: prothymocytes enter the thymus via vessels localized at the cortico-medullary junction where CCL21 and CXCL12 seem to play an important role in their recruitment [89]. Progression of thymocytes within the thymus is then orchestrated through the interplay of different chemokines, with CXCL12 playing a role in the proper localization of early progenitors into the cortex [89]. CCL25 produced by TECs and medullary DCs, interacts with CCR9 that is up-regulated following pre-TCR signaling on $CD4^+CD8^+$ -double-positive thymocytes and also on $CD4^+$ - or $CD8^+$ -single-positive thymocytes [106,107]. Double-positive thymocytes are also responsive to CCL22, via CCR4 [108]. CCL19 and CCL21 act via a common receptor, CCR7, whose expression is observed on pro-thymocytes but also on $CD4^+$ - or $CD8^+$ -single-positive thymocytes. CCR7 plays a role in positive selection by directing the migration of thymocytes into the thymic medulla [109–111]. Other chemokines, such as CXCL9, CXCL10, and CXCL11 (via CXCR3) are also produced by medullary TECs and participate in the migration of diverse thymocyte subsets [88]. CCL22 and CXCL12 are expressed by TECs cells in the medulla and might play a role in negative selection by inducing apoptosis of thymocytes [104,108]. Finally, two chemokines seem to be particularly involved in the export of thymocytes to the periphery through a chemorepulsive effect (chemorepulsion) [112]. CCL19 is produced by medullary TECs and, especially, in TECs surrounding medullary vessels. Apparently, CCL19 plays a key role in migration of mature thymocytes that have survived the process of negative selection into the periphery [108,111]. CXCL12 is also implicated in chemorepulsive action contributing to the egress of mature SP $CD4^+$ cells from the fetal thymus [113]. (B) Role of chemokines in thymic hyperplasia: in the thymus of MG patients, abnormal expression and localization of chemokines contribute to peripheral-cell trafficking leading to thymic hyperplasia. In particular, overexpression of the B-cell chemoattractant CXCL13 by TECs could explain the generalized thymic B-cell infiltration observed in all MG patients [31]. In hyperplastic MG thymuses, CCL21 overexpression due to lymphatic vessels might also play a central role in peripheral cell trafficking in and/or out the thymus [39]. CXCL12, whose level of expression does not change in the thymus of MG compared to non-MG patients, is nevertheless abnormally localized on certain thymic vessels (unpublished data), suggesting also its involvement in peripheral cell recruitment to MG thymus.

CCL21 and CCL19

CCR7 ligands, CCL21 and CCL19, play an important role in thymopoiesis (Figure 3(A)). In the normal thymus, they are mainly secreted by medullary TECs and promote the migration of CD4⁺ or CD8⁺ thymocytes toward the medullary zone [88]. They also play distinct roles due to their different presentation patterns on distinct stromal components: CCL21 participates in prothymocyte recruitment in the thymus [89], whereas CCL19 is involved in the export of mature thymocytes toward the periphery [90]. Comparison of the thymic CCL21/CCL19 ratios indicated a major representation of CCL19 in babies, which might be related to the high thymic cell mass and the need to export mature thymocytes to the periphery. Accordingly, in involuted adult thymuses, the level of CCL19 decreased [39].

Thymic hyperplasia in MG patients is specifically associated with the thymic overexpression of CCL19 and, in particular, of CCL21, albeit without variations of CCR7 expression on peripheral lymphocytes or thymic expression of CCR11, a decoy receptor for these two chemokines [39]. The high level of CCL21 compared to CCL19 could lead to changes in the patterns of thymocyte recruitment and export. In this context, these changes could explain the increased number of CD4⁺ observed in MG thymuses [91].

In periphery, CCL21 is known to play a central role in immune surveillance and defense by controlling the circulation of T cells and DCs within lymphoid and peripheral organs. In SLOs, CCL21 enables naïve T cells to encounter sensitized DCs by mediating their recruitment through its expression on HEVs and afferent lymphatic vessels, respectively [92]. We demonstrated that CCL21 overexpression in hyperplastic MG thymuses is due to lymphatic endothelial vessels that could correspond to specialized efferent lymphatic vessels involved in the export of differentiated T and B cells emerging from GCs. This route of circulation for differentiated T cells is observed not only in SLOs, but also in peripheral organs [93]. However, the thymic CCL21-positive lymphatic endothelial vessels in MG could also correspond to afferent lymphatic vessels as they express D6 [39], considered as a marker for these vessels [42]. Consequently, CCL21-positive lymphatic vessels could lead to an abnormal recruitment of sensitized DCs, as suggested by the work of Nagane et al. [44] who showed the presence of activated DCs around vessels in hyperplastic MG thymuses. It could also lead to a recruitment of peripheral cells, and indeed, we showed that CCL21 was a potent chemoattractant for T and B cells, especially for naïve B cells [39].

CXCL10

CXCL10, also named IFN- γ -induced protein 10 (IP10), binds to CXCR3 and regulates immune responses through activation and recruitment of leukocytes, such as T cells, eosinophils, monocytes, and natural killer cells [94]. CXCL10 is increased in various autoimmune diseases, where it seems to play a role in the infiltration of inflammatory sites by peripheral cells [95]. In normal thymus, CXCL10 is produced by medullary TECs and participate in the migration of diverse thymocyte subsets [88]. In MG, an increased thymic expression of CXCL10 and its receptor CXCR3 was demonstrated together with a significant increase in CXCR3 on peripheral CD4⁺ T cells, suggesting a recruitment of these cells to the thymus of MG patients [96]. Although CXCL10 has been described on HEVs in SLOs [97], we did not observe any expression of CXCL10 on thymic HEVs in MG patients (data not shown). The increased expression of CXCL10 in MG patients could also affect the role of medullary TECs in thymopoiesis. Indeed, it has recently been observed that CXCL10 can induce the production of receptor activator for nuclear factor κ B ligand (RANKL) by CD4⁺T cells [98], which regulates mTEC development [99,100] and thereby modulates self-tolerance.

CXCL12

CXCL12, also known as stromal-derived factor-1 (SDF-1), was shown to contribute to B- and T-cell homing and GC organization [101,102]. In the thymus (Figure 3(A)), CXCL12 has been described at the cortico-medullary junction, in the cortex, and in medullary areas including Hassall's bodies [103,104]. Expression of CXCR4, the receptor for CXCL12, has been clearly observed on early progenitors in normal thymus [105]. In MG, we have not observed any increased expression of CXCL12 in the thymus [31] or in cultures of TECs from MG patients (data not shown). However, our recent data have indicated that there is an abnormal localization of CXCL12 on certain thymic vessels (manuscript in preparation), suggesting that CXCL12 might also participate in the recruitment of peripheral cells in the thymus of MG patients, leading to thymic hyperplasia.

CCL5/RANTES

CCL5 is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. There is apparently an increased expression of CCL5 in the hyperplastic thymus of MG patients [44], and CCL5 levels are abnormally augmented in TECs from MG compared to non-MG patients upon induction by adhesion-related stimuli [48]. In MG patients, CCL5 could promote the recruitment of peripheral T and B

cells to the thymus and favor GC development. However, further work is required to define the possible involvement of this chemokine in thymic hyperplasia.

Conclusions

Defining what is happening in the thymus, the effector organ in MG, should help understand the etiology/pathogenesis of this disease. The triggering events leading to thymic abnormalities in MG patients are not yet clearly defined, but seem related to the inflammatory signature characteristic of MG thymuses. Inflammatory molecules then trigger an aberrant expression pattern of chemokines that play a central role in recruiting peripheral cells to the thymus, where, in the inflammatory environment characteristic of MG thymuses, they participate in the sensitization against AChR.

Thymic increases in CXCL13 [22], CCL21 [39], and CXCL10 [96] are normalized in glucocorticoid-treated patients, suggesting that pharmacological drugs blocking chemokine–receptor interactions could prevent the development of thymic hyperplasia in MG, thereby abrogating the need for thymectomy and the use of non-specific glucocorticoid treatments.

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IV. GENERAL CONCLUSION

While the autoantigenic target and the immune effector mechanisms are well defined in MG, the events leading to the breakdown of self-tolerance to AChR remain a mystery. Histological, clinical and immunological evidence strongly indicate that the thymus holds the keys to solve this enigma. The thymus of early onset MG patients contains all the components necessary for an anti-AChR immune response including autoreactive B and T cells, APCs as well as the AChR and displays a hyperplasia characterized by features of SLOs with B-cell infiltration, GC development and HEV formation. These hallmarks support the idea of a causal relationship between the thymus and MG.

To better understand the development of thymic hyperplasia, the aim of my thesis was to analyze the role of chemokines in the recruitment of peripheral cells to the thymus. In SLOs, cell recruitment is mediated by HEVs, which correspond to an entrance gate for peripheral cells. In the MG thymus, I demonstrated that HEV development correlated with the degree of thymic hyperplasia and that the number of HEVs was reduced in MG patients under corticotherapy. Corticosteroid treatment is known to decrease the number of GCs in the hyperplastic thymus [176], which could be a consequence of the diminished number of HEVs. When we analyzed the peripheral cells that localized around HEVs, we detected B cells, pDCs, mDCs and monocytes/macrophages. The recruitment of B cells via HEVs was not surprising, as the MG thymus contains GCs, but the recruitment of the other peripheral cells had not been clearly shown before. For the first time, we described that pDCs, mDCs and monocytes/macrophages could migrate from the periphery to the MG thymus via HEVs and settle in their vicinity.

As cell migration to SLOs is induced by chemokines displayed on the lumen of HEVs, we investigated the presence of chemokines on thymic HEVs in MG. We focused on chemokines that are known to be expressed on HEVs in SLOs and that had been found to be upregulated in the thymus of MG patients. As Méraouna *et al.* described elevated levels of CXCL13 in epithelial cells of MG thymus [176], we analyzed CXCL13 expression on HEVs using immunofluorescence; however, we were not able to locate it on thymic HEVs [270]. CCL21 was also reported to be overproduced in hyperplastic MG

thymus due to its expression by lymphatic vessels [171], but we were not able to detect its presence on HEVs. Other chemokines such as CXCL10 [263], CCL19 [177], CXCL9 and CXCL11, which were also described to be overexpressed in the MG thymus and/or upregulated in a transcriptome study of our group, were not found to be displayed on the HEV surface, either. The only chemokine that we localized on the lumen side of HEVs was SDF-1. We therefore investigated, if it could be involved in the HEV-mediated peripheral cell recruitment of APCs and B cells. As SDF-1 attracts cells by binding to its receptor CXCR4, we analyzed the CXCR4 expression on peripheral cells around HEVs. We found that some of the B cells and APCs that located around and inside HEVs expressed CXCR4 indicating that SDF-1 navigated these cells from the periphery to the thymus. In parallel, we observed a diminished CXCR4 expression on peripheral mDCs and a decreased number of mDCs and monocytes in the blood from MG patients suggesting a recruitment of these cells. In patients under cortico-therapy, which reduces the numbers of HEVs, the changes in the periphery were normalized suggesting a link to the hyperplastic thymus. Altogether, we showed that thymic HEVs act as a portal for APCs and B cells entering into the MG thymus and identified the SDF-1/CXCR4 interplay as a mechanism by which the peripheral cell recruitment is mediated. With SDF-1 on HEVs, we thus detected a new factor participating in thymic hyperplasia, that could represent a new attractive therapeutical target.

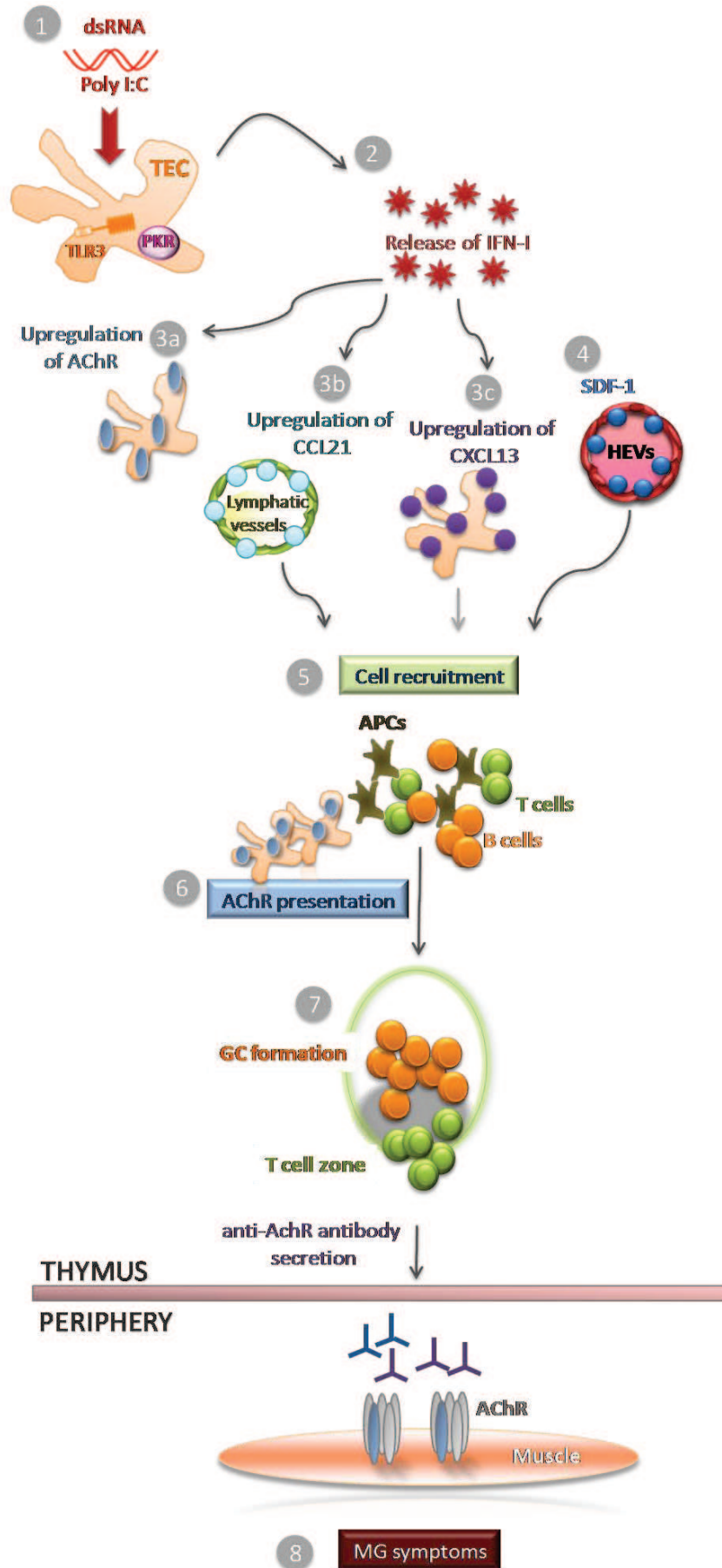
The mechanism by which ectopic HEVs develop is not precisely known. Studies in mice showed that overexpression of CXCL13 in the pancreas resulted in the formation of HEVs and in CCL21 upregulation. As mentioned earlier CXCL13 and CCL21 are overexpressed in MG thymus and could therefore contribute to the angiogenic processes. So far their role in MG is defined as mediators of cell recruitment: CCL21, overexpressed by lymphatic vessels in MG thymus, could contribute to thymic hyperplasia development by recruiting T cells and especially naïve B cells [171]. CXCL13, a very potent B-cell attractant, also seems to be implicated in navigating peripheral cells to the MG thymus, as chemotactic experiments of thymic extracts from MG had a strong effect on B cells [176]. This effect was impaired when using anti-CXCL13 antibodies. However, how CXCL13 can guide peripheral cells to the thymus without being expressed on HEVs remains unclear.

To define the role of CXCL13 and to test as a proof of principle if it was sufficient to mediate B cell recruitment to the thymus, we generated a transgenic mouse model with a CXCL13 overexpression in TECs. Our preliminary results showed that the overexpression of CXCL13 in transgenic K5-CXCL13 mice induced an increased number of cells in the thymus. When we analyzed the cell composition in the thymus of K5-CXCL13 mice, we observed an augmented number of B cells, which preferentially settled in loose aggregates in medullary regions. These observations prove that CXCL13 overexpression by TECs can mediate B-cell recruitment to the thymus. Even though we induced B-cell migration to the thymus, the increased presence of B cell did not seem to trigger GC formation. It is likely that the establishment of thymic hyperplasia requires additional immunomodulating factor(s). This factor could be CCL21, as some chronic autoimmune diseases have demonstrated that the formation of GCs required both CXCL13 and CCL21 engagement [257],[271].

Investigating molecules that could be involved in chemokine overexpression in the MG thymus, we demonstrated that IFN-I could be a central role in MG development. Indeed, transcriptome studies had demonstrated an IFN-I signature in MG thymus with upregulation of numerous IFN-I-induced genes [174]. Analyzing the effect of IFN-I on cultured TECs and lymphatic endothelial cells (LECs), we demonstrated the upregulation of CXCL13 and CCL21 in TECs and in LECs, respectively (Cufi *et al.*, in preparation). This upregulation was inhibited, when cells were pre-treated with corticosteroids. IFN-I is known to be induced by signaling through TLR, which are receptors for pathogen-associated molecules. When we tested the effect of several TLR agonists on the regulation of IFN-I on human TECs, we observed that the TLR3 agonist Poly(I:C), a dsRNA analogue, induced the production of thymic IFN-I associated with an overexpression of α -AChR. When we injected wildtype mice with Poly(I:C), we found elevated levels of IFN-I and α -AChR, as well as CXCL13 and CCL21 in the thymus. These inductions were not observed in mice deficient for the IFN-I receptor indicating that Poly(I:C) effects are mediated through the release of IFN-I. In parallel, we also observed the development of an autoimmune response against AChR in the periphery.

Altogether, the results obtained during my PhD contribute to a better understanding of the mechanisms that lead to thymic hyperplasia and MG. The events induced by signaling of dsRNA, CXCL13 and SDF-1, which are part of a larger scenario including additional factors, can be summarized as follows:

1. On the top of the cascade stands an initial event that induces the activation of dsRNA pathways. This event could be a viral infection or the release of molecules from necrotic or apoptotic cells. Other predisposing factors are probably also important in this initiating phase, such as the genetic background related to the MHC genes or a hormonal imbalance.
2. dsRNA activates pathogen-signaling pathways and triggers the release of IFN-I. Other cytokines upregulated in MG could also intervene and modulate or amplify IFN-I effects, such as IFN-II.
3. IFN-I upregulates the expression of α -AChR and chemokines including CCL21 in lymphatic endothelial cells and CXCL13 in TECs.
4. CXCL13 or other yet unknown factors could promote the formation of HEVs expressing SDF-1.
5. mDCs, pDCs, monocytes/macrophages, B cells and T cells enter the thymus through HEVs and also through lymphatic vessels.
6. Sensitization to AChR could then occur: One possibility is that an increased death of cells that express AChR, such as TECs or myoid cells in this inflammatory environment stimulates antigen uptake by APCs. APCs could then process and cross-present autoantigen peptides to B and T cells, which initiates the anti-AChR immune response.
7. B cells and T cells start to get organized in lymphoid structures and develop GCs leading to thymic hyperplasia.
8. Autoreactive B cells lead to the production of high-affinity anti-AChR antibodies that can bind to AChR on muscles in the periphery and mediate MG symptoms.



V. PERSPECTIVES

The understanding of the pathophysiological mechanisms leading to thymic hyperplasia is indispensable for the proposition of novel therapies. With CXCL13 and SDF-1, but also CCL21, we have identified new potential targets, whose specific inhibition could block the abnormal peripheral cell recruitment to the thymus and consequently hyperplasia. In chronic inflammatory diseases, chemokines and their receptors present attractive therapeutic targets to diminish inflammation and strategies neutralizing the effect of chemokines are currently under investigation. Antagonists for CCR1, CCR2, CXCR3 and CCR9 are at present tested in Phase II or III trials on patients of multiple sclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease and Crohn's disease, respectively [218],[217]. Inhibition of SDF-1 or its ligands appears to be rather complex, as SDF-1 is involved in many homeostatic processes demonstrated by the fact that SDF-1 and CXCR4 knockout mice are lethal in the embryonic state [272],[273]. Nevertheless, investigations on NZB/W mice, a mouse model of systemic lupus erythematosus, injected with neutralizing anti-SDF-1 antibodies showed that disease development could be prevented by inhibition of autoantibody production [274]. Treatment with CXCR4 antagonist of another murine lupus model achieved similar results [275]. CXCR4 antagonist also reduced disease severity in experimental autoimmune encephalitis (EAE), an animal model for multiple sclerosis, and collagen-induced arthritis (CIA). Inhibition of CXCR4 diminished CD4⁺T cell infiltration into the central nervous system (CNS) in EAE and blocked leukocyte migration to the joints in mice with CIA [276],[277]. The benefit of an SDF-1/CXCR4 blocking therapy in MG patients is arguable, since we observed a decrease in SDF-1 serum levels in MG (data not shown). Moreover, unlike in systemic lupus erythematosus, multiple sclerosis or RA, where SDF-1 is overexpressed [275],[278],[279], SDF-1 in MG is not upregulated, but it is the development of thymic HEVs expressing SDF-1 that leads to peripheral cell recruitment. Consequently, it might be more advisable to inhibit the SDF-1-mediated cell recruitment by acting on the angiogenic processes in the thymuses and by preventing HEV development. However, little data

exist on angiogenic inhibitors specific for HEV formation. The soluble LT β R immunoglobulin fusion protein, an antagonist of membrane lymphotoxin- β , is so far the only molecule proposed to reduce HEV-development [238, 280].

Research on neutralizing CXCL13 is more advanced and the effects of CXCL13 inhibition on cell infiltration in inflammatory diseases are presently under investigations: Gardner *et al.* have demonstrated that anti-CXCL13 and anti-TNF α combination therapy in NZB/W mice reduced lymphocyte infiltration and B cell proliferation in the kidney and, thereby, decreased the titer of pathogenic serum auto-antibodies [281]. In a mouse model for arthritis, neutralization of CXCL13 reduced the number of ectopic GCs in arthritic joints and thus impaired the severity of CIA. In order to test the effect of CXCL13 inhibition on thymic hyperplasia in MG, an EAMG model with a hyperplastic thymus is required. We are therefore currently investigating, if the administration of torpedo-AChR in complete Freud's adjuvant to transgenic K5-CXCL13 mice can result in EAMG with thymic hyperplasia. We also plan to test the potential of Poly(I:C) injections to trigger hyperplasia. An EAMG model with a hyperplastic thymus will give us the opportunity to test whether CXCL13 or CXCR5 antagonists are able to decrease the number of GCs and MG symptoms. However, we could also test other pharmacological compounds in an EAMG model, which represent the human disease better than the classical EAMG model.

The long-term goal of the research that I developed during my PhD was to better understand the thymic pathophysiology of MG in order to find new therapeutical targets that could block the abnormal recruitment of peripheral cells in the thymus. These drugs could prevent thymic hyperplasia development for MG patients avoiding thymectomy and the use of non-specific glucocorticoids. The results of this research could be also beneficial for other autoimmune diseases, especially those characterized by the development of ectopic GCs.

VI. REFERENCES

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RÉSUMÉ

La myasthénie (Myasthenia Gravis) est une maladie neuromusculaire impliquant des auto-anticorps dirigés majoritairement contre le récepteur à l'acétylcholine (RACH) et entraînant une fatigabilité musculaire. Ces auto-anticorps pathogènes sont produits principalement par le thymus qui présente une hyperplasie caractérisée par le développement de centres germinatifs ectopiques. De récentes études ont démontré la surexpression de chimiokines dans le thymus des patients et la présence anormale de vaisseaux sanguins de type HEV (cellules endothéliales à paroi haute). **L'objectif de ma thèse a été de mieux comprendre les mécanismes physio-pathologiques conduisant à l'hyperplasie thymique en étudiant le rôle des chimiokines dans la myasthénie.**

Nous avons tout d'abord démontré que le nombre de HEV thymiques est proportionnel au degré d'hyperplasie suggérant leur implication directe dans le recrutement des cellules périphériques. En analysant les chimiokines exprimées sur ces HEV, nous observons l'expression sélective de SDF-1/CXCL12. En parallèle, la présence de lymphocytes B, de cellules dendritiques myéloïdes ou plasmacytoïdes et de monocytes/macrophages exprimant le récepteur au SDF-1, CXCR4, a été observée au niveau des HEV. En périphérie, nous montrons une diminution de l'expression de CXCR4 ainsi que du nombre de mDC et de monocytes dans le sang des patients suggérant le recrutement de ces cellules dans le thymus.

Le thymus des patients myasthéniques est aussi caractérisé par une surexpression de la chimiokine CXCL13 par les cellules épithéliales thymiques. Pour mieux comprendre les mécanismes conduisant à l'hyperplasie thymique, nous avons développé un modèle de souris transgéniques avec surexpression thymique de CXCL13. Dans le thymus de ces souris, nous observons une surexpression de CXCL13 et une augmentation de nombre de lymphocytes B, notamment pour les souris jeunes. Nous étudions maintenant si l'immunisation de ces souris avec du RACH purifié induit une myasthénie expérimentale associée à une hyperplasie thymique ; un nouveau modèle animal de la maladie qui se rapprocherait mieux de la pathologie humaine.

Dans la myasthénie, le thymus est aussi caractérisé par une signature inflammatoire, avec notamment une surexpression d'interféron de type I (IFN-I). Nous démontrons que le Poly(I:C), une molécule mimant les effets des ARN double-brin, induit spécifiquement la surexpression du RACH- α par les cellules épithéliales thymiques humaines via la libération d'IFN-I. L'IFN-I entraîne aussi la surexpression des chimiokines CXCL13 et CCL21 comme dans le thymus des patients myasthéniques. Chez des souris C57Bl6, mais pas chez des souris KO pour le récepteur à l'IFN-I, des injections de Poly(I:C) entraînent des modifications thymiques avec une surexpression spécifique de RACH- α , d'IFN-I et de chimiokines. En périphérie, ces injections entraînent l'apparition dans le sérum d'anticorps contre le RACH- α spécifiques de la myasthénie.

L'ensemble de ces résultats suggère que dans le thymus des patients myasthéniques, le développement anormal de HEV exprimant du SDF-1 et la surexpression de CXCL13 joueraient un rôle central dans le recrutement de cellules périphériques. Ces cellules, une fois dans l'environnement inflammatoire caractéristique du thymus myasthénique, pourraient alors développer une réaction auto-immune contre le RACH. De nouvelles molécules thérapeutiques contrôlant l'expression de ces chimiokines ou l'angiogenèse pourraient diminuer le développement de l'hyperplasie thymique et éviteraient la thymectomie ou l'utilisation des glucocorticoïdes par les patients atteints de myasthénie.