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

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ABSTRACT

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

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

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

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

1. Introduction

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

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

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Abbreviations

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

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

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

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

2. Material and methods

2.1. Thymic biopsies

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

2.2. Human serum and blood cells

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

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

2.3. Primary cell cultures

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

2.4. Co-culture assays

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

2.5. Quantitative real-time PCR

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

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

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

(*continued on next page*)

Table 1 (*continued*)

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

summarized in Table 2.

2.6. ELISA

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

2.7. Flow cytometry analyses

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

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

2.8. Immunofluorescence microscopy

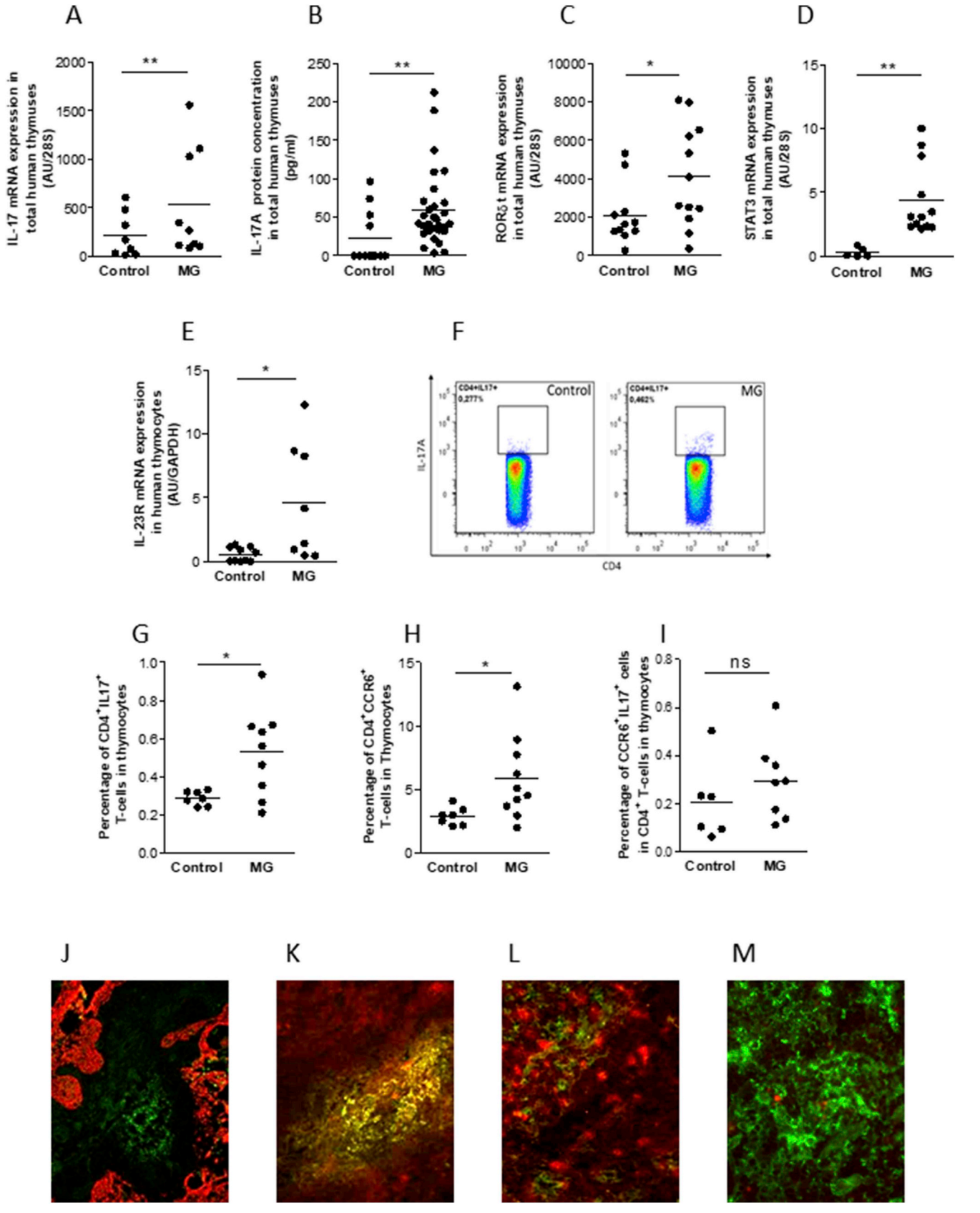
Cryostat sections (7 μm) of frozen human thymic tissues were fixed with acetone to glass superfrost slides and dried for 1 h. The human thymic sections were pre-incubated with a blocking buffer (PBS, 0.1% BSA, 10% FBS, 0.3 M Glycine, 1%Tween) for 1 h at room temperature and then, incubated overnight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

2.9. Statistical analysis

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

Table 2
List of primers used in the study.

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



(caption on next page)

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

3. Results

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

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

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

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

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

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

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

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

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

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

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

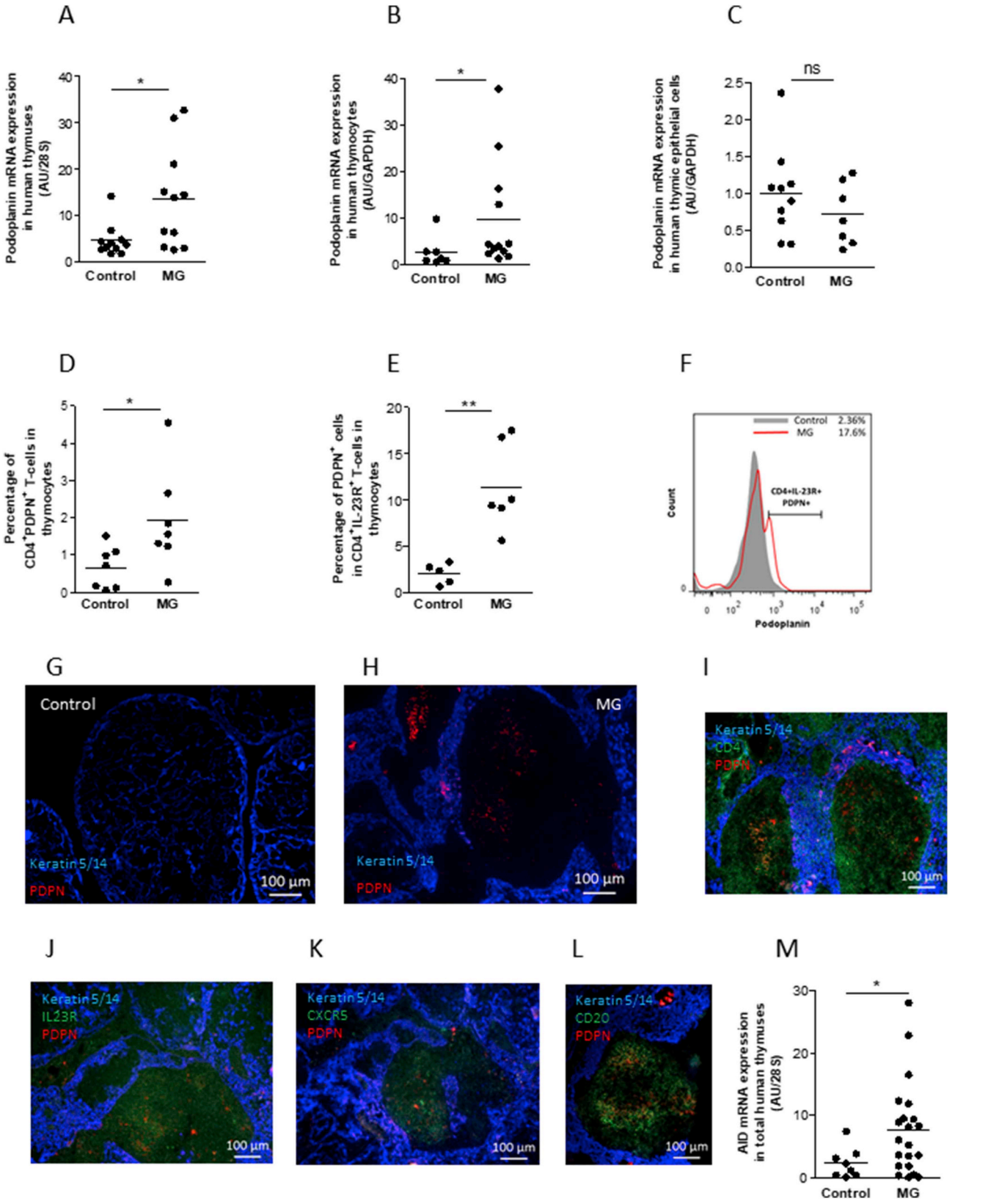


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Fig. 2. Podoplanin expression in AChR$^+$ MG thymuses. Comparative analysis of podoplanin mRNA expression in **A)** global thymic biopsies, **B)** purified human thymocytes and **C)** primary cultured thymic epithelial cells obtained from AChR$^+$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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

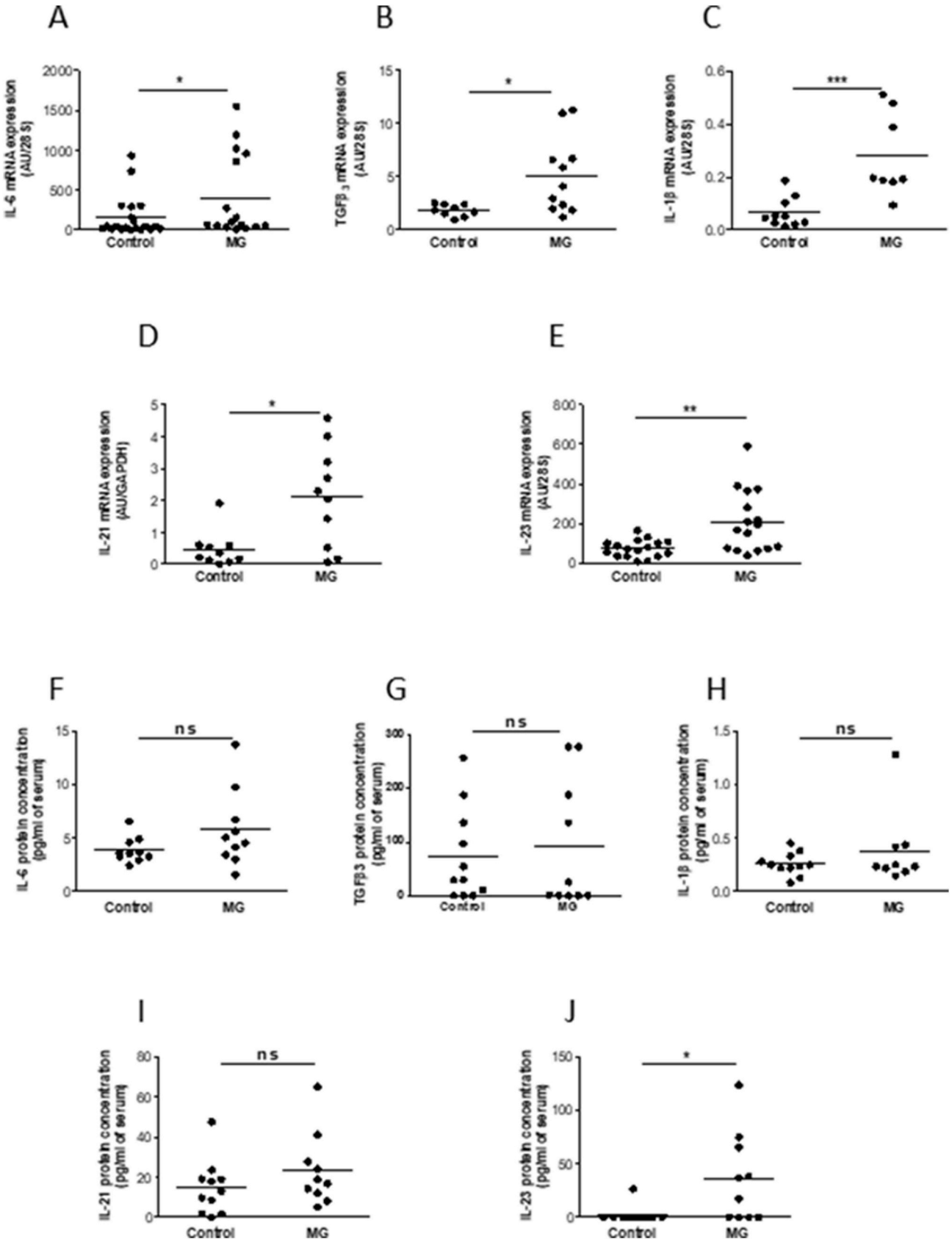

Fig. 3. Expression of cytokines involved in Th17 cells differentiation in thymuses of AChR$^+$ MG patients. mRNA expression levels of **A)** IL-6, **B)** TGF-β3, **C)** IL-1β, **D)** IL-21, and **E)** IL-23 in thymuses of AChR$^+$ MG patients compared to aged matched control adults. Protein expression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

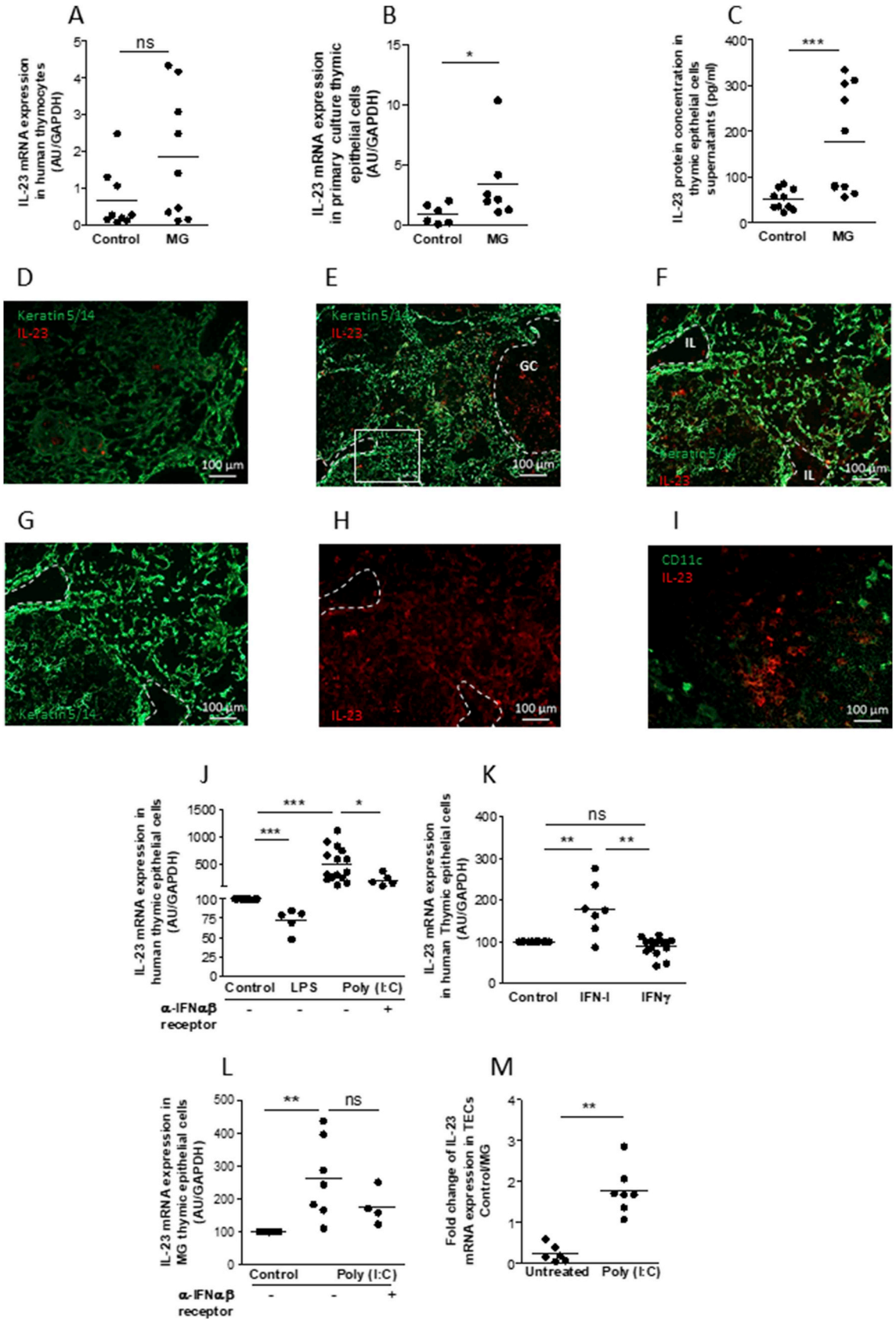

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Fig. 4. Overexpression of IL-23 is due to "inflamed" TECs in thymuses of MG patients. Analysis of mRNA expression level of IL-23 in **A)** thymocytes and **B)** primary cultured TECs of AChR$^+$ MG patients compared to controls. **C)** Protein expression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

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

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

4. Discussion

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

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

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

4.1. MG TECs support thymic inflammation through Th17 cell activation

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

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

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

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

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

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

4.3. MG TECs contribution in eGC formation

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

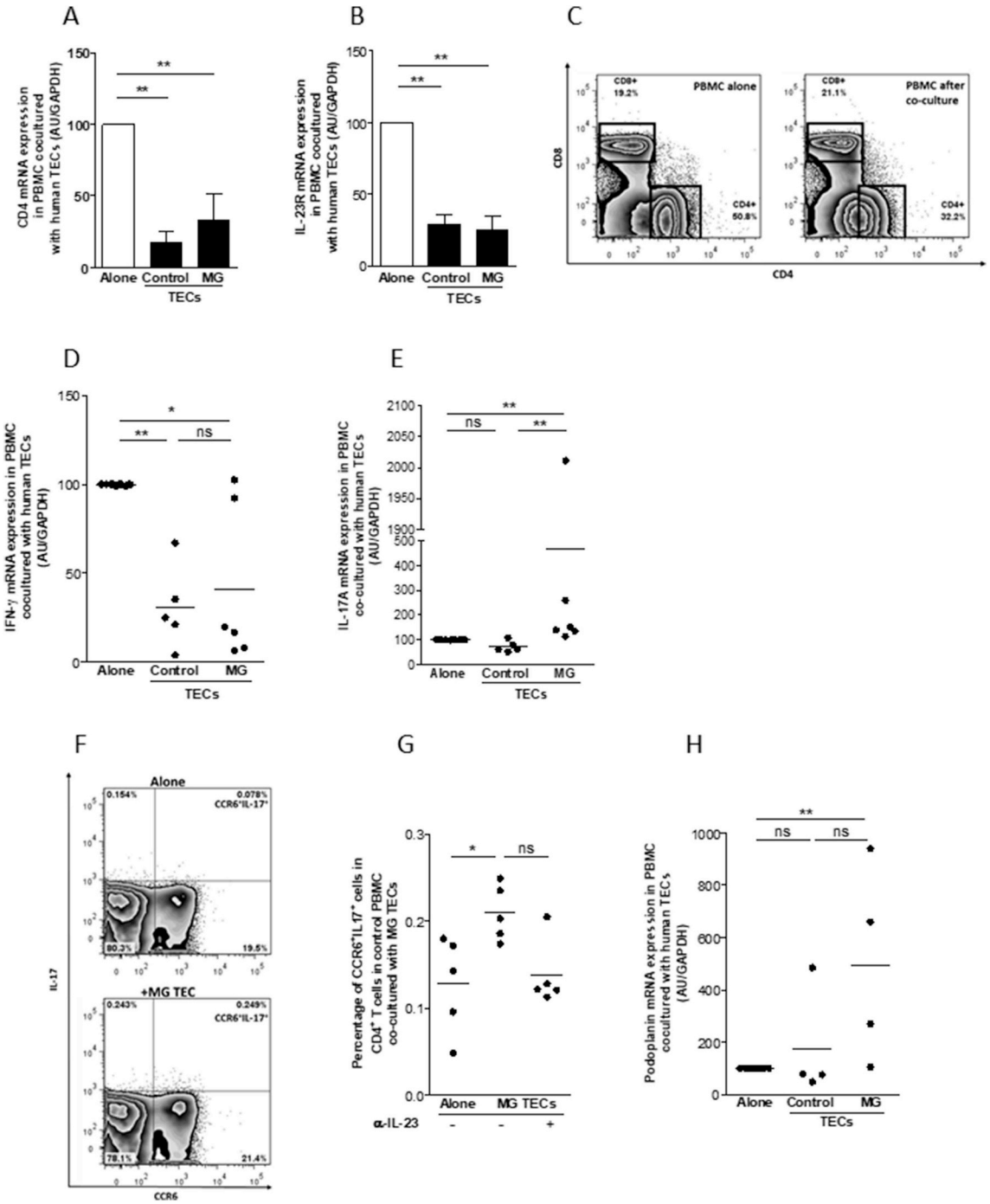

Fig. 5. MG TEC secretion of IL-23 stimulates IL-17 production by PBMCS. Analysis of the mRNA expression of **A)** CD4 and **B)** IL-23 receptor in PBMCs co-cultured for 24 h with controls or AChR$^+$ MG primary TECs. **C)** Representative images of flow cytometry analysis of CD4 and CD8 labelling of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

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

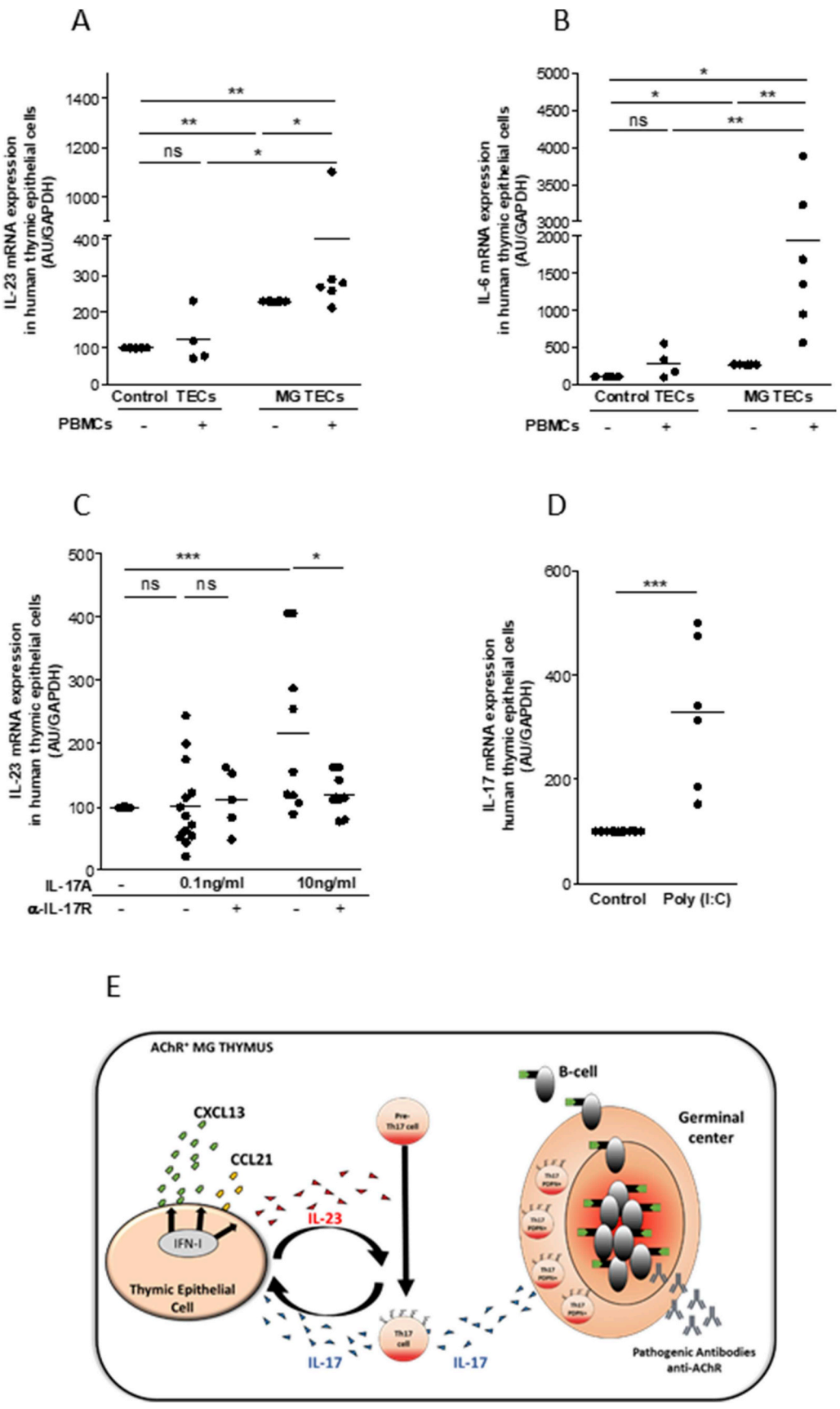

Fig. 6. PBMCs co-cultured with TECs influence the TEC expression of cytokines. mRNA expression level of **A)** IL-23 and **B)** IL-6 in control and MG AChR$^+$ TECs co-cultured with control PBMCs. **C)** Effect of IL-17 (0.1 ng/ml and 10 ng/ml) and anti-IL-17 receptor (α-IL-17R) (1 μg/ml) on the mRNA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

4.4. Why does the inflammatory process do not stop?

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

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

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

5. Conclusions

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

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

Authors contributions

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

Declaration of interest

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

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References

[1] S. Berrih-Aknin, R. Le Panse, Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms, J. Autoimmun. 52 (2014) 90–100.
[2] M. Sudres, M. Maurer, M. Robinet, J. Bismuth, F. Truffault, D. Girard, et al., Preconditioned mesenchymal stem cells treat myasthenia gravis in a humanized preclinical model, JCI Insight 2 (2017) e89665.
[3] G.I. Wolfe, H.J. Kaminski, I.B. Aban, G. Minisman, H.C. Kuo, A. Marx, et al., Randomized trial of thymectomy in myasthenia gravis, N. Engl. J. Med. 375 (2016) 511–522.
[4] R. Le Panse, G. Cizeron-Clairac, J. Bismuth, S. Berrih-Aknin, Microarrays reveal distinct gene signatures in the thymus of seropositive and seronegative myasthenia gravis patients and the role of CC chemokine ligand 21 in thymic hyperplasia, J. Immunol. 177 (2006) 7868–7879.
[5] R. Le Panse, J. Bismuth, G. Cizeron-Clairac, J.M. Weiss, P. Cufi, P. Dartevelle, et al., Thymic remodeling associated with hyperplasia in myasthenia gravis, Autoimmunity 43 (2010) 401–412.
[6] A. Balandina, S. Lecart, P. Dartevelle, A. Saoudi, S. Berrih-Aknin, Functional defect of regulatory CD4(+)CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis, Blood 105 (2005) 735–741.
[7] M. Thiruppathi, J. Rowin, B. Ganesh, J.R. Sheng, B.S. Prabhakar, M.N. Meriggioli, Impaired regulatory function in circulating CD4(+)CD25(high)CD127(low/-) T cells in patients with myasthenia gravis, Clin. Immunol. 145 (2012) 209–223 Orlando, Fla.
[8] A. Gradolatto, D. Nazzal, F. Truffault, J. Bismuth, E. Fadel, M. Foti, et al., Both Treg cells and Tconv cells are defective in the Myasthenia gravis thymus: roles of IL-17 and TNF-alpha, J. Autoimmun. 52 (2014) 53–63.
[9] Y. Xie, H.F. Li, B. Jiang, Y. Li, H.J. Kaminski, L.L. Kusner, Elevated plasma interleukin-17A in a subgroup of Myasthenia Gravis patients, Cytokine 78 (2016) 44–46.
[10] A. Meraouna, G. Cizeron-Clairac, R.L. Panse, J. Bismuth, F. Truffault, C. Tallaksen, et al., The chemokine CXCL13 is a key molecule in autoimmune myasthenia gravis, Blood 108 (2006) 432–440.
[11] H. Schaffert, A. Pelz, A. Saxena, M. Losen, A. Meisel, A. Thiel, et al., IL-17-producing CD4(+) T cells contribute to the loss of B-cell tolerance in experimental autoimmune myasthenia gravis, Eur. J. Immunol. 45 (2015) 1339–1347.
[12] M. Milani, N. Ostlie, H. Wu, W. Wang, B.M. Conti-Fine, CD4+ T and B cells cooperate in the immunoregulation of experimental autoimmune myasthenia gravis, J. Neuroimmunol. 179 (2006) 152–162.
[13] M.S. Maddur, P. Miossec, S.V. Kaveri, J. Bayry, Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies, Am. J. Pathol. 181 (2012) 8–18.
[14] A. Peters, L.A. Pitcher, J.M. Sullivan, M. Mitsdoerffer, S.E. Acton, B. Franz, et al., Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation, Immunity 35 (2011) 986–996.
[15] Y. Miyamoto, H. Uga, S. Tanaka, M. Kadowaki, M. Ikeda, J. Saegusa, et al., Podoplanin is an inflammatory protein upregulated in Th17 cells in SKG arthritic joints, Mol. Immunol. 54 (2013) 199–207.
[16] A. Peters, P.R. Burkett, R.A. Sobel, C.D. Buckley, S.P. Watson, E. Bettelli, et al., Podoplanin negatively regulates CD4+ effector T cell responses, J. Clin. Investig. 125 (2015) 129–140.
[17] D. Nazzal, A. Gradolatto, F. Truffault, J. Bismuth, S. Berrih-Aknin, Human thymus medullary epithelial cells promote regulatory T cell generation by stimulating interleukin-2 production via ICOS ligand, Cell Death Dis. 5 (2014) e1420.
[18] J.A. Villegas, A. Gradolatto, F. Truffault, R. Roussin, S. Berrih-Aknin, R. Le Panse, et al., Cultured human thymic-derived cells display medullary thymic epithelial cell phenotype and functionality, Front. Immunol. 9 (2018) 1663.
[19] N. Dragin, J. Bismuth, G. Cizeron-Clairac, M.G. Biferi, C. Berthault, A. Serraf, et al., Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases, J. Clin. Investig. 126 (2016) 1525–1537.
[20] P. Cufi, P. Soussan, F. Truffault, R. Fetouchi, M. Robinet, E. Fadel, et al., Thymoma-associated myasthenia gravis: on the search for a pathogen signature, J. Autoimmun. 52 (2014) 29–35.
[21] G. Piskin, R.M. Sylva-Steenland, J.D. Bos, M.B. Teunissen, In vitro and in situ expression of IL-23 by keratinocytes in healthy skin and psoriasis lesions: enhanced expression in psoriatic skin, J. Immunol. 176 (2006) 1908–1915.
[22] M. Mitsdoerffer, Y. Lee, A. Jager, H.J. Kim, T. Korn, J.K. Kolls, et al., Proinflammatory T helper type 17 cells are effective B-cell helpers, Proceedings of the National Academy of Sciences of the United States of America, vol. 107, 2010, pp. 14292–14297.
[23] J.T. Gaublomme, N. Yosef, Y. Lee, R.S. Gertner, L.V. Yang, C. Wu, et al., Single-cell genomics unveils critical regulators of Th17 cell pathogenicity, Cell 163 (2015)

1400–1412.
[24] A. Vaknin-Dembinsky, K. Balashov, H.L. Weiner, IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production, J. Immunol. 176 (2006) 7768–7774.
[25] P. Cufi, N. Dragin, J.M. Weiss, P. Martinez-Martinez, M.H. De Baets, R. Roussin, et al., Implication of double-stranded RNA signaling in the etiology of autoimmune myasthenia gravis, Ann. Neurol. 73 (2013) 281–293.
[26] I.V. Grishkan, A. Ntranos, P.A. Calabresi, A.R. Gocke, Helper T cells down-regulate CD4 expression upon chronic stimulation giving rise to double-negative T cells, Cell. Immunol. 284 (2013) 68–74.
[27] R. Stadhouders, E. Lubberts, R.W. Hendriks, A cellular and molecular view of T helper 17 cell plasticity in autoimmunity, J. Autoimmun. 87 (2018) 1–15.
[28] J.L. Astarita, S.E. Acton, S.J. Turley, Podoplanin: emerging functions in development, the immune system, and cancer, Front. Immunol. 3 (2012) 283.
[29] S. Cohen-Kaminsky, C. Gaud, E. Morel, S. Berrih-Aknin, High recombinant interleukin-2 sensitivity of peripheral blood lymphocytes from patients with myasthenia gravis: correlations with clinical parameters, J. Autoimmun. 2 (1989) 241–258.
[30] S. Cohen-Kaminsky, R.M. Delattre, O. Devergne, I. Klingel-Schmitt, D. Emilie, P. Galanaud, et al., High IL-6 gene expression and production by cultured human thymic epithelial cells from patients with myasthenia gravis, Ann. N. Y. Acad. Sci. 681 (1993) 97–99.
[31] P. Cavalcante, R. Le Panse, S. Berrih-Aknin, L. Maggi, C. Antozzi, F. Baggi, et al., The thymus in myasthenia gravis: site of “innate autoimmunity”? Muscle Nerve 44 (2011) 467–484.
[32] M. Colombara, V. Antonini, A.P. Riviera, F. Mainiero, R. Strippoli, M. Merola, et al., Constitutive activation of p38 and ERK1/2 MAPKs in epithelial cells of myasthenic thymus leads to IL-6 and RANTES overexpression: effects on survival and migration of peripheral T and B cells, J. Immunol. 175 (2005) 7021–7028.
[33] D. Emilie, O. Devergne, S. Cohen-Kaminsky, S. Berrih-Aknin, P. Galanaud, In situ production of interleukins in hyperplastic thymus from myasthenia gravis patients, Ann. N. Y. Acad. Sci. 681 (1993) 100–102.
[34] P. Bernasconi, L. Passerini, A. Annoni, F. Ubiali, C. Marcozzi, P. Confalonieri, et al., Expression of transforming growth factor-beta1 in thymus of myasthenia gravis patients: correlation with pathological abnormalities, Ann. N. Y. Acad. Sci. 998 (2003) 278–283.
[35] Y. Lee, A. Awasthi, N. Yosef, F.J. Quintana, S. Xiao, A. Peters, et al., Induction and molecular signature of pathogenic TH17 cells, Nat. Immunol. 13 (2012) 991–999.
[36] E. Bettelli, T. Korn, M. Oukka, V.K. Kuchroo, Induction and effector functions of T(H)17 cells, Nature 453 (2008) 1051–1057.
[37] C. Aime, S. Cohen-Kaminsky, S. Berrih-Aknin, In vitro interleukin-1 (IL-1) production in thymic hyperplasia and thymoma from patients with myasthenia gravis, J. Clin. Immunol. 11 (1991) 268–278.
[38] M.W. Teng, E.P. Bowman, J.J. McElwee, M.J. Smyth, J.L. Casanova, A.M. Cooper, et al., IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases, Nat. Med. 21 (2015) 719–729.
[39] A.L. Croxford, F. Mair, B. Becher, IL-23: one cytokine in control of autoimmunity, Eur. J. Immunol. 42 (2012) 2263–2273.
[40] C.L. Langrish, Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, et al., IL-23 drives a pathogenic T cell population that induces autoimmune inflammation, J. Exp. Med. 201 (2005) 233–240.
[41] W. Wang, M. Milani, N. Ostlie, D. Okita, R.K. Agarwal, R.R. Caspi, et al., C57BL/6 mice genetically deficient in IL-12/IL-23 and IFN-gamma are susceptible to experimental autoimmune myasthenia gravis, suggesting a pathogenic role of non-Th1 cells, J. Immunol. 178 (2007) 7072–7080.
[42] D.J. Cua, J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, et al., Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain, Nature 421 (2003) 744–748.
[43] M. Kleinewietfeld, D.A. Hafler, The plasticity of human Treg and Th17 cells and its role in autoimmunity, Semin. Immunol. 25 (2013) 305–312.
[44] P.R. Burkett, zu Meyer, G. Horste, V.K. Kuchroo, Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity, J. Clin. Investig. 125 (2015) 2211–2219.
[45] Y. Li, C. Wei, H. Xu, J. Jia, Z. Wei, R. Guo, et al., The immunoregulation of Th17 in host against intracellular bacterial infection, Mediat. Inflamm. 2018 (2018) 6587296.
[46] P. Cufi, N. Dragin, N. Ruhlmann, J.M. Weiss, E. Fadel, A. Serraf, et al., Central role of interferon-beta in thymic events leading to myasthenia gravis, J. Autoimmun. 52 (2014) 44–52.
[47] S. Poea-Guyon, P. Christadoss, R. Le Panse, T. Guyon, M. De Baets, A. Wakkach, et al., Effects of cytokines on acetylcholine receptor expression: implications for myasthenia gravis, J. Immunol. 174 (2005) 5941–5949.
[48] N. Dragin, P. Nancy, J. Villegas, R. Roussin, R. Le Panse, S. Berrih-Aknin, Balance between estrogens and proinflammatory cytokines regulates chemokine production involved in thymic germinal center formation, Sci. Rep. 7 (2017) 7970.
[49] D.C. Newcomb, J.Y. Cephus, M.G. Boswell, J.M. Fahrenholz, E.W. Langley, A.S. Feldman, et al., Estrogen and progesterone decrease let-7f microRNA expression and increase IL-23/IL-23 receptor signaling and IL-17A production in patients with severe asthma, J. Allergy Clin. Immunol. 136 (2015) 1025–1034 e11.
[50] S. Berrih-Aknin, Myasthenia Gravis: paradox versus paradigm in autoimmunity, J. Autoimmun. 52 (2014) 1–28.
[51] H.C. Hsu, P. Yang, J. Wang, Q. Wu, R. Myers, J. Chen, et al., Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice, Nat. Immunol. 9 (2008) 166–175.
[52] H.J. Koenen, R.L. Smeets, P.M. Vink, E. van Rijssen, A.M. Boots, I. Joosten, Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells, Blood 112 (2008) 2340–2352.
[53] F. Fossiez, O. Djossou, P. Chomarat, L. Flores-Romo, S. Ait-Yahia, C. Maat, et al., T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines, J. Exp. Med. 183 (1996) 2593–2603.
[54] S.L. Gaffen, R. Jain, A.V. Garg, D.J. Cua, The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing, Nat. Rev. Immunol. 14 (2014) 585–600.
[55] J. Frieder, D. Kivelevitch, I. Haugh, I. Watson, A. Menter, Anti-IL-23 and anti-IL-17 biologic agents for the treatment of immune-mediated inflammatory conditions, Clin. Pharmacol. Therapeut. 103 (2018) 88–101.