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Characterization of the thymus in Lrp4 myasthenia gravis: Four cases

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A B S T R A C T

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction. Most patients have pathogenic autoantibodies against the acetylcholine receptor (AChR). In the last years a novel subpopulation of MG patients has been described that harbors antibodies against low-density lipoprotein receptor-related protein 4 (Lrp4), another postsynaptic neuromuscular antigen. In early-onset AChR MG (EOMG), the thymus plays an important role in immunopathogenesis, and early thymectomy is beneficial. It is still unknown if the thymus plays any role in Lrp4-MG. In this pilot study, we compared thymus samples from four patients with Lrp4-MG (one pre-treated with immunosuppressive drugs), four non-MG controls and five EOMG patients (not pretreated with immunosuppressive drugs). Immunohistochemistry of the Lrp4-MG thymi revealed normal architecture, with normal numbers and distribution of B-cells, lymphoid follicles and Hassall's corpuscles. Primary $CD23^+$ lymphoid follicles were similarly infrequent in Lrp4-MG and control thymic sections. In none of the control or Lrp4-MG thymi did we find secondary follicles with $CD10^+$ germinal centers. These were evident in 2 of the 5 EOMG thymi, where primary lymphoid follicles were also more frequent on average, thus showing considerable heterogeneity between patients. Even if characteristic pathological thymic changes were not observed in the Lrp4 subgroup, we cannot exclude a role for the thymus in Lrp4-MG pathogenesis, since one Lrp4-MG patient went into clinical remission after thymectomy alone (at one year follow-up) and one more improved after thymectomy in combination with immunosuppressive therapy.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction (NMJ) characterized by fatigable skeletal muscle weakness. Approximately 85% of patients harbor autoantibodies against the muscle acetylcholine receptor (AChR) and 5–8% of the patients have autoantibodies to muscle specific kinase (MuSK) [1–3]. Antibodies against low-density lipoprotein receptor-like protein 4 (Lrp4) were recently discovered, and found in 2–50% of the patients with no detectable antibodies against either MuSK or AChR [4–7], but also in a low proportion of patients with AChR MG or MuSK MG, which was then associated with severe weakness [4,6–9]. Lrp4-MG patients have a female predominance (2.5:1 F:M sex ratio) and present clinically mostly with mild generalized muscle weakness, in some cases with bulbar or ocular involvement.

AChR MG consists of different subgroups, namely early- or late-onset MG (EOMG or LOMG, onset before or after the age of 50 years), thymoma-associated MG [10,11,13], ocular MG (reviewed in [12]). Many EOMG thymi contain numerous secondary lymphoid follicles in extra-parenchymal infiltrates [14–16]. Both are much less frequent in healthy individuals, where primary lymphoid follicles have been noted in a third of control thymi [17]; they are small, inactive and widely scattered (3–4 per microscopic field at 1.4× magnification) [18]. In EOMG, by contrast, they contain germinal centers with Bcl-6 and

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$CD10^+$ cells [19], and are widely implicated in the production of AChR autoantibodies [14,20–25]. Importantly, removing the thymus is clinically beneficial and is an established treatment in EOMG [26,27]. In contrast to EOMG, the thymus in MuSK MG is normal in most reported cases [28]. Its possible involvement in Lrp4-MG pathogenesis demands evaluation, as it might hint at potential benefits of thymectomy. In this small exploratory study, we looked for morphological abnormalities in four Lrp4-MG thymi. Specifically, we quantitated primary lymphoid follicles using CD23 as an established marker of follicular dendritic cell networks [19,29] to find evidence for follicular hyperplasia, and secondary follicles using CD10 as a marker of germinal center specific B- and T follicular helper cells [30–32]. In addition, we investigated key cell populations that are implicated in EOMG pathogenesis [19,20,24], including B-cells, plasma cells, myoid cells, thymic epithelial cells and T-cells.

2. Materials and methods

2.1. Patients

The diagnosis of MG was based on clinical signs and symptoms, electrophysiology (SFEMG/RNS) and/or a clinical response to pyridostigmine. Five patients had antibodies against AChR and 4 against Lrp4, and none had both or MuSK antibodies (Table 1). They were thymectomized as part of standard management. The four age-matched non-MG controls were undergoing cardiac surgery. Only one Lrp4-MG patient (L4) was taking any immunosuppressive medication (methylprednisolone and azathioprine; Table 1). All samples were taken with informed consent and local Ethics Committee approval.

Three of the Lrp4-MG thymic specimens, one control thymus and all five EOMG thymic specimens were obtained from the Maastricht Pathology Tissue Collection, one Lrp4-MG thymus was derived from Antwerp Medical Center. Three control thymi were provided by the Institute of Pathology of the University Medical Centre Mannheim. All use of tissue and patient data from the Dutch patients was in agreement with the Dutch Code of Conduct for Observational Research with Personal Data (2004) and Tissue (2001, Federatie van Medisch Wetenschappelijke Verenigingen (FMWV)).

The EOMG thymus samples were selected from the Maastricht Pathology Tissue Collection based on the following criteria: no immunosuppressive, steroid or IVIg treatment before thymectomy and no evidence of neoplasms (including thymoma). Initially, 66 patients matching these criteria were identified, of whom 49 were diagnosed with 'follicular hyperplasia' based on T and B cell staining. The remaining patients were diagnosed with:- 'non-involuted thymus', 'lymphoid hyperplasia' or 'thymic remnant'. The five EOMG thymus samples were selected to represent this diversity. The clinical data of the patients included in this study are summarized in Table 1.

2.2. Autoantibody diagnosis

Autoantibodies against AChR and MuSK were determined by routine RIA and ELISA; against Lrp4 using cell-based assays with fixed and permeabilized cells as described [7], and verified by live cell-based assay [33] in patient L1.

2.3. Immunohistochemistry

We mounted sections of paraffin-embedded (3 μm) or frozen thymus (10 μm) on superfrost plus microscopic slides. Due to limited availability of tissue, one section was used per staining. We stained with these antibodies (all from Dako) against established markers: a) "Ready to use": anti-CD20 (mature B-cells, clone L26), anti-CD23 (follicular dendritic cells, clone DAK-CD23), anti-CD10 (germinal center B cells, clone: 56C6) anti-CD3 (T-cells, polyclonal) anti-CD4 (T helper cells, clone 4B12), anti-CD8 (cytotoxic T-cells, clone C8/144B), anti-cytokeratin AE1/AE3 (thymic epithelial cells (TEC), clone AE1/AE3), anti-desmin (myoid cells, clone D33), anti-CD138 (plasma cells, clone MI15), and b) anti-Igκ, anti-Igλ, (plasma cells, polyclonal, dilution 1:40,000), anti- CD35 (DAKO, dilution 1:25). The immunohistochemical stainings were conducted on a Dako Autostainer Link48 according to standard diagnostic routine protocols and manufacturers' instructions.

2.4. Semiquantitative analysis of primary and secondary follicles

Microscopic scans of whole tissue sections were acquired using a Ventana iScan HT scanner (Roche). Both for microscopic slide scans and individual photomicrographs, a BX50 microscope with a DP70 camera (Olympus), a motorized stage (Märzhäuser) and Stereoinvestigator stereology software (Microbrightfield) were used.

The numbers of primary CD23+ follicles or CD10+ germinal centers within secondary follicles were counted manually in the scans. For each primary follicle, the diameter was measured and only follicles that were within the previously published range of 20–430 μm [34] were included. The numbers were normalized to the tissue area measured using Ventana software.

2.5. Thymus cell isolation and culture

Fresh thymus was available from one Lrp4-MG patient (L1). Tissue was dispersed as described [35]. Briefly, it was cut into cubes of ~1 mm^3, and digested with 10 mg/ml collagenase from *Clostridium histolyticum* (catalogue number C6885, Sigma) and 10 U/ml nuclease from *Staphylococcus aureus* (catalogue number N3755, Sigma, 100–300 units/mg protein), at 37 °C one hour, vortexing every 10 min. The cells were then passed through a 100 μm sieve, washed with PBS and each 6×10^6 cells/well were cultured in round bottom 96 wells in the presence or absence of 10 μg/ml pokeweed mitogen (PWM, catalogue number L9379-5MG, Sigma). In parallel, PBMCs from peripheral blood of the Lrp4 patient were isolated and cultured at 6×10^5 cells/well in the presence or absence of 10 μg/ml PWM. After one week in culture, 90 μl of the medium was removed and replaced by 100 μl fresh medium every three to four days; the IgG concentration in the supernatant was analyzed by ELISA (as described [36]) at days 8, 12, 15 and 18.

2.6. Statistical analysis

Due to the small sample number and the heterogeneity within the three disease groups (degree of involution, steroid treatment in Lrp4-MG patients) statistical analysis was not considered appropriate.

3. Results

3.1. Clinical outcome after thymectomy

We noted that myasthenic symptoms ameliorated after thymectomy in two Lrp4-MG patients (Table 1): Patient L1 went into complete clinical remission without any further treatment; and patient L3 improved from MGFA IIIA to I on corticosteroids. Ocular muscle weakness of patient L2 before thymectomy was still present at 3 years after thymectomy. The one patient L4 who had received methylprednisolone and azathioprine prior to thymectomy has no clinical follow up yet.

3.2. Thymus tissue architecture and density of germinal centers

We next tested our initial hypothesis – that the thymus might contribute to the pathogenesis of Lrp4-MG – by comparing its architecture and defined cell subsets with four age-matched non-MG controls and five representative EOMG thymi. We found (i) as expected, varying degrees of thymus involution in all subgroups (Table 1 and

Table 1
Clinical data.

		Non-MG controls				EOMG	
	Patient code	C1	C2	C3	C4	A1	A2
At time of thymectomy	Gender	F	M	M	F	F	M
	Age (in years)	26	27	37	30	23	16
	Treatment					Pyridostigmine	Pyridostigmine
	MG duration (months)					30	13
	Symptoms					Ptosis, diplopia, limb and jaw weakness	Ptosis, diplopia
	MGFA score	0	0	0	0	IVB	I
	Thymus histology	Non-involuted, slightly increased	Partially involuted	Mostly involuted	Mostly involuted	Non-involuted with follicular hyperplasia	Non-involuted
At follow-up	Treatment						
	MGFA (time post-Tx, months)					IIA (24)	

	EOMG			Lrp4 MG			
	A3	A4	A5	L1	L2	L3	L4
At time of thymectomy	F	F	F	M	M	F	F
	34	29	38	28	38	40	53
	Pyridostigmine	Pyridostigmine	Pyridostigmine		Pyridostigmine	Pyridostigmine	Methylprednisolone, azathioprine
	5	8	18	4	21	25	> 1
	Ptosis, bulbar and limb weakness	Bulbar, hand and limb weakness	Bulbar weakness, ptosis,diplopia	limb weakness, dysphagia	Ocular weakness	Ptosis, limb weakness	Limb and voice weakness
	IIB	IIIB	IIB	IIB	I	IIIA	IIA
	Partially involuted with follicular hyperplasia	Non-involuted with follicular hyperplasia	Mostly involuted	Partially involuted	Partially involuted	Partially involuted	Mostly involuted
At follow-up					Azathioprine	Corticosteroids	
	IIB (1)		0 (17)	0 (12)	I (36)	IIA (8); I (19)	

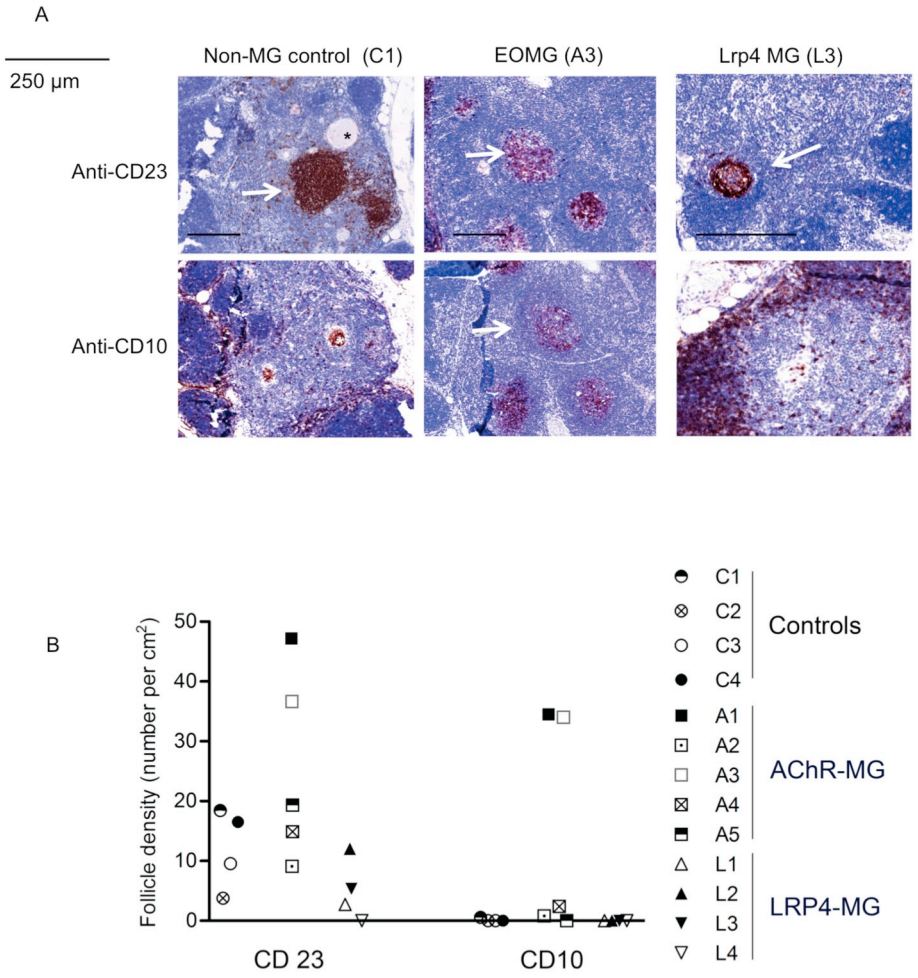

Fig. 1. Analysis of primary and secondary lymphoid follicles. Lymphoid follicles were stained with anti-CD23 and germinal centers in secondary follicles were stained with CD10. A) Serial sections from biopsies from non-MG control, EOMG and Lrp4-MG thymus were stained with anti-CD23 or anti-CD10. Asterisks indicate the presence of Hassall's corpuscles, arrows indicate examples of primary or secondary follicles. B) Density of primary and secondary lymphoid follicles. Whole tissue scans were obtained and the number of $CD23^+$ and $CD10^+$ lymphoid follicles per tissue area were analyzed.

Supplemental Fig. 1), and densely packed B-cells near the Hassall's corpuscles within the medulla (Fig. 2A) (ii) Cytokeratin-negative areas of extra-parenchymal infiltrates in the EOMG thymi with primary (Figs. 1B, 2A, Supplementary Figs. 1, 2) and secondary lymphoid follicles (Fig. 1B). In contrast, primary follicles were less common and germinal centers absent in Lrp4-MG and control thymi (Figs. 1, 2, Supplementary Figs. 1, 2); the observed follicles were in the extra-parenchymal space (cytokeratin negative areas in the medulla).

We next investigated the histology (Fig. 1A) and density (Fig. 1B) of primary lymphoid follicles by staining for CD23 and CD35 on follicular dendritic cells (Supplementary Fig. 2). We also quantitated secondary follicles in adjacent thymus sections by staining for CD10, a marker of germinal center-specific B- and T follicular helper cells (Fig. 1B). The Lrp4-MG thymus samples were characterized by low numbers of primary lymphoid follicles, similar to the non-MG controls. Neither non-MG controls nor Lrp4-MG patients had any secondary, $CD10^+$ lymphoid follicles. Notably, in the two EOMG patients with the highest number of $CD23^+$ lymphoid follicles, most of them contained $CD10^+$ germinal centers.

In the Lrp4 thymi, we next focused on markers for other relevant cell populations that are known to be of pathogenic relevance for EOMG [20,24], namely Igκ, Igλ and CD138 (plasma cell markers), CD3 (T-cells), desmin (myoid cells), and CD20 (B-cells). While thymi in EOMG showed the characteristic accumulations of $CD20^+$ B cells and $CD3^+$ mature T cells in expanded perivascular spaces with displacement and disruption of their $keratin^+$ epithelial cell lining [37–39], these were not obvious in Lrp4 thymi. $Desmin^+$ myoid cells and $Igκ^+$ plasma cells that typically occur in the vicinity of lymphoid follicles in EOMG thymi [40] were regularly located around Hassall's corpuscles, as in non-MG control thymi.

In the EOMG thymus, antigen-specific long-lived plasma cells can be found that produce total IgG and AChR autoantibodies *in vitro* without PWM stimulation [35]. Fresh thymus cells isolated and cultured from patient L1 produced 2 µg/ml IgG *in vitro* both in the presence and absence of PWM while PBMCs of the same patient only produced 0.5 µg/ml IgG when stimulated with PWM (data not shown). However, we could not detect Lrp4 autoantibodies in the supernatants by cell-based assay.

4. Discussion

Autoantibodies against Lrp4 were recently discovered in MG patients without antibodies against MuSK or AChR [4–7]. Thymic abnormalities (but no thymomas) were reported in a subset of Lrp4-MG patients [7]. Here, we undertook an in-depth analysis of thymectomy specimens of four clinically well-characterized Lrp4-MG patients (three of them corticosteroid treatment-naïve). In short, our findings of an unaltered thymic architecture, normal or even slightly reduced numbers of primary lymphoid follicles, B-cells and FDC networks, absence of germinal centers and the failure to detect autoantibodies in Lrp4

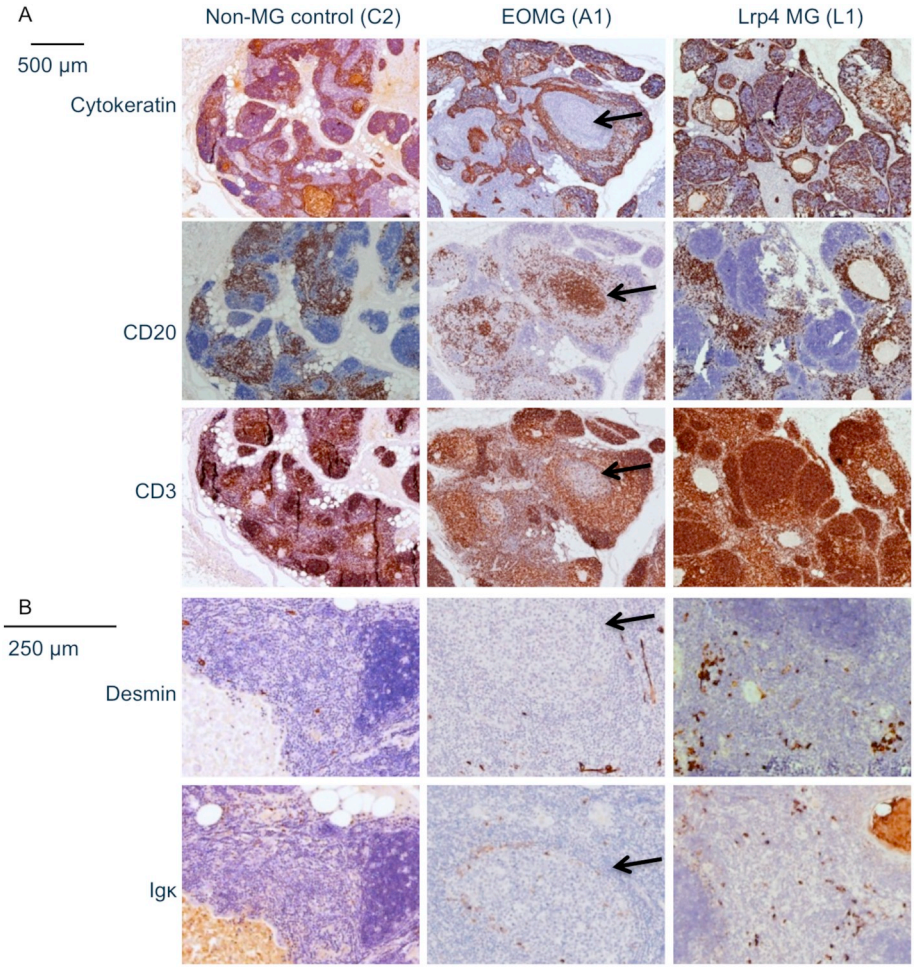

Fig. 2. Immunohistochemistry analysis of key thymic cell subsets in Lrp4-MG, EOMG and control thymi. These contain B-cells ($CD20^+$), epithelial cells (pan cytokeratin), T-cells ($CD3^+$), myoid cells ($desmin^+$) and plasma cells ($Igκ^+$). Left column: Non-MG control thymus, middle column: EOMG thymus and right column: Lrp4-MG thymus. Arrow indicates an exemplary lymphoid follicle. A) Images acquired at 4× magnification. Scale bar = 500 µm. B) Box selection from A as indicated, scale bar = 250 µm. Antibody stainings as indicated = DAB/brown. Hematoxylin = blue.

thymic culture supernatants, are in sharp contrast to what is characteristic for thymic follicular hyperplasia in EOMG [14,19,25,35,41,42]. However the Lrp4-MG thymi appeared heterogeneous, with thymic abnormalities in only 32% of patients, in a large epidemiological study [7]. Although this argues against our original hypothesis that there could be a similar intrathymic pathogenesis of Lrp4-MG as observed in EOMG, two out of three of the Lrp4 patients with available follow-up information showed clinical improvement after thymectomy This apparent discrepancy does not disprove the clinical usefulness of thymectomy in the management of Lrp4-MG – which warrants further studies of thymectomy and thymus histology and function, *e.g.* dysregulation of chemokines, cytokines or miRNAs [43], to extend this pilot screen of four of these rare cases of Lrp4-MG.

5. Conclusion

In this pilot study, thymus specimens of four Lrp4-MG patients were investigated for the first time according to recently proposed criteria [27] and state-of-the art techniques. No indication for intrathymic pathogenesis as hypothesized for EOMG could be found, but the clinical improvement of two patients after thymectomy (one in absence of any immunosuppression) advises further studies to assess clinical usefulness of thymectomy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autrev.2018.07.011.

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