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Research article

Stereotypic T cell receptor clonotypes in the thymus and peripheral blood of Myasthenia gravis patients

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ABSTRACT

Myasthenia Gravis (MG) patients with anti-acetylcholine receptor (AChR) antibodies frequently show hyperplastic thymi with ectopic germinal centers, where autoreactive B cells proliferate with the aid of T cells. In this study, thymus and peripheral blood (PB) samples were collected from ten AChR antibody-positive MG patients. T cell receptor (TCR) repertoires were analyzed using next-generation sequencing (NGS), and compared with that of an age and sex matched control group generated from a public database. Certain V genes and VJ gene recombination pairs were significantly upregulated in the TCR chains of αβ-T cells in the PB of MG patients compared to the control group. Furthermore, the TCR chains found in the thymi of MG patients had a weighted distribution to longer CDR3 lengths when compared to the PB of MG patients, and the TCR beta chains (TRB) in the MG group's PB showed increased clonality encoded by one upregulated V gene. When TRB sequences were sub-divided into groups based on their CDR3 lengths, certain groups showed decreased clonality in the MG group's PB compared to the control group's PB. Finally, we demonstrated that stereotypic MG patient-specific TCR clonotypes co-exist in both

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the PB and thymi at a much higher frequency than that of the clonotypes confined to the PB. These results strongly suggest the existence of a biased T cell-mediated immune response in MG patients, as observed in other autoimmune diseases.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease at the neuromuscular junctions, and is clinically characterized by the fluctuating weakness of skeletal muscles. Symptoms of MG include diplopia, ptosis, dysarthria, difficulty in swallowing, and weakness in the limbs. Antibodies against the acetylcholine receptor (anti-AChR antibody) are detected in approximately 70% of MG patients and have been suggested to be the pathogenic [1]. Research has shown the accumulation of somatic hypermutations and class-switching in anti-AChR antibodies, thus indicating the presence of T cells specifically reactive to the acetylcholine receptor (AChR) [2,3]. These T cells were identified in the peripheral blood (PB) of MG patients [3], and were found to proliferate and trigger a pro-inflammatory response when exposed to AChR [4]. T cells reactive to AChR were also observed in the thymi of MG patients [5], which are known to be the site where autoreactive B cells are produced and proliferate. Recently, the role of T cells on the pathogenesis of MG has been further supported by the following findings: IFN-γ and IL-7 are produced by CD4 T cells when stimulated with AChR peptide, production of cytokine and anti-AChR antibody is decreased in IL-17 knock-out mice, and regulatory T cells are decreased in number or functionally impaired in patients with MG [6].

Although T cells play an important role in the pathogenesis of MG and recent reports showed that analyses of T cell receptor (TCR) repertories could provide further understanding of autoimmune diseases [7–9], the immunologic profile of autoreactive T cells in MG patients has been less thoroughly investigated compared to those of B cells. T cells are classified into two types (αβ-T cells and γδ-T cells), depending on the type of TCR displayed on the cell surface. TCRαβ is a heterodimer protein expressed on the surface of T cells, and recognizes antigen-derived peptides complexed with the human leukocyte antigen (HLA) molecule. Both the TCRα chain (TRA) and TCRβ chain (TRB) are encoded by the variable (V) and joining (J) genes, with the additional diversity (D) gene assembled between the V and J genes in TRB. The recombination of these genes along with random or palindromic nucleotide insertions provide enormous diversity to TCR repertoires [10]. However, under conditions where the immune system is chronically exposed to specific antigens, as in autoimmune disease, the TCR repertoires demonstrate conserved TCR sequences or restricted Vβ gene usage [8,9]. Recent studies have also demonstrated that analyzing TCR repertoires in autoimmune diseases can serve diagnostic and prognostic purposes [8,9].

In the present study, we aimed to assess the characteristics of TCR repertoires in the PB and thymi of MG patients by using high-throughput next generation sequencing (NGS). In addition, we analyzed the stereotypic TCR clonotypes specifically and commonly found in the PB and thymi of MG patients.

2. Materials and methods

2.1. Study design and sample collection

This study was designed to investigate the distinct characteristics of TCR repertoires in patients with MG. Thymic tissue and peripheral blood were retrieved from a pre-existing biorepository that collected thymic tissue paired with peripheral blood mononuclear cells (PBMCs) from MG patients who underwent thymectomy. Both samples were collected on the day of the thymectomy. The following inclusion criteria was used: 1) patients with anti-AChR antibody-positive MG, and 2) patients whose clinical features of MG and treatment status were recorded. MG diagnosis was made by confirming clinical symptoms, a positive response to the neostigmine test, decrement responses on repetitive nerve stimulation, and the presence of anti-AChR antibodies. Finally, 10 thymi and 10 paired PBMCs were collected from MG patients and included for analysis. For controlled comparison with healthy subjects, 10 age/sex-matched healthy individuals were chosen from a publicly available database containing information about their TCR repertoires [11–17], all of which were constructed from PB samples. For all collected samples, TRA and TRB transcripts were amplified and subjected to NGS to construct TCR repertoires. TCR repertoires were characterized by the usage of V genes and VJ gene recombination pairs, distribution of CDR3 length, clonality, and the existence and abundance of stereotypic TCRs specific to MG patients. This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University Health System (IRB number: 4-2023-0685).

2.2. NGS library preparation

PBMC were isolated from patient blood samples using Ficoll gradients (GE Healthcare, Chicago, IL, USA), and the total RNA was extracted using TRIzol reagent (15,596,018; Invitrogen, La Jolla, CA, USA) according to the manufacturer's protocol. Thymic tissue was collected from MG patients who underwent thymectomy to treat MG or thymoma. In patients without thymoma, 1–5 g of thymic tissue was collected. In patients with thymoma, 1–5 g of non-neoplastic thymic tissue adjacent to the thymoma was collected. Thymic tissue was homogenized on ice in 1 mL TRIzol (Invitrogen, Waltham, MA, USA), added with 0.2 ml of chloroform, incubated for 5 min, and centrifuged at 12,000×*g* at 4 °C for 15 min. The aqueous phase was carefully separated, added to 0.5 ml of 100% isopropanol, incubated for 10 min, and centrifuged at 12,000×*g* at 4 °C for 15 min. After discarding the supernatant, the pellet was washed with 1 mL of 75% ethanol per 1 mL of TRIzol, and centrifuged at 12,000×*g* at 4 °C for 15 min. The supernatant was discarded, and the RNA pellet was air-dried for 8 min, then resuspended in 20 μL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Waltham, MA,

USA). 1 μg of total RNA was used to synthesize the complementary DNA (cDNA), using the SuperScript IV First-Strand Synthesis System (Invitrogen) with specific primers targeting the constant region (Supplementary Table 1). After synthesis of the first-strand cDNA, 1.8 vol of SPRI beads (AMPure XP, Beckman Coulter, Brea, CA, USA) were used to purify the cDNA, which was then eluted in 35 μL of water. The purified cDNA (15 μL) was subjected to second-strand synthesis in a 25 μL reaction volume using KAPA Biosystems (KAPA HiFi HotStart, Roche, Basel, Switzerland) with V gene-targeting primers (Supplementary Table 1). The PCR conditions were as follows: 95 °C for 3 min; 1 cycle for TRB and 4 cycles for TRA of 98 °C for 30 s, 60 °C for 40 s, 72 °C for 1 min; and 72 °C for 5 min. After synthesis of the second-strand, the double stranded DNA (dsDNA) was purified using 1.0 volume of SPRI beads. Genes were amplified using 15 μL of purified dsDNA in a 25 μL total reaction volume (KAPA Biosystems) with universal primers (Supplementary Table 1), using the thermal cycling program: 95 °C for 3 min; 25 cycles of 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. Amplicons were gel purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany). Gel purified amplicons were further purified using 1.0 volume of SPRI beads, quantified with a 4200 TapeStation System (Agilent Technologies), and subjected to NGS using Illumina Novaseq platform.

2.3. Processing of NGS data for the TCR repertoires

The raw paired-end fastq files were processed to extract functional reads of TCR repertoires as previously described [18], using the updated version of IgBLAST (v1.17.1) for defining the V(D)J regions (VJ gene, CDRs). Sequences composed of the same V(D)J regions and unique molecular identifier (UMI) from different sample sources were eliminated to null out artifacts generated during sequencing, such as molecular contamination or index misassignments. The contamination-eliminated and functional TCR sequences were defined as productive reads, and subjected to further analysis, including repertoire characterization and investigation of stereotypic clonotypes.

2.4. Characterization of TCR repertoires

From the TCR repertoires of MG patients and an age/sex-matched healthy control group, we investigated the V gene and VJ gene recombination pair usage, CDR3 length distribution, and TCR clonality using an in-house python script (v3.6). The VJ gene and CDR3 region of each TCR were defined using IgBLAST (v1.17.1), and clonality was measured by the Gini index (0–1), where a value near 1 represents a higher abundance of select TCR clones within the entire repertoire, and 0 represents equal abundance of all TCR clones. Stereotypic TCR clonotypes were defined as the collection of TCR sequences containing identical VJ genes and CDR3 amino acids in more than one MG patient. Stereotypic MG patient-specific TCR clonotypes were defined by excluding the clonotypes that were also found in the datasets of healthy subjects [11–17]. These clonotypes were then sub-divided into groups based on their localization; those found only in the PB, only in the thymus, or in both the PB and thymus respectively. The number and frequency of stereotypic MG patient-specific TCR clonotypes were computed for each group.

2.5. Statistical analysis

All data comparing the characteristics between two groups (PB of MG patients and an age/sex-matched healthy control group, or thymi and PB of MG patients) were statistically analyzed by student t-test or Mann-Whitney *U* test, depending on the normality and equal-variance of the data sets. The statistical tests were performed using SciPy library (v.1.5.4) in python. Significance was denoted by the number of asterisks from one to four, each representing $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$.

3. Results

3.1. Clinical characteristics of MG patients

Ten MG patients were included in the present analysis. Clinical characteristics of the included patients are summarized in Supplementary Table 2. The median (Q1 – Q3) age was 36.0 (31.5–48.0) years. One patient was diagnosed with ocular MG and the remaining nine patients were diagnosed with the generalized form of MG. Thymic hyperplasia was observed in two patients and thymoma was found in in eight patients. All patients were positive for anti-AChR antibody with a median titer of 15.8 (5.6–17.4) nmol/L. Three patients were on corticosteroid treatment.

3.2. MG patients possess biased usage of V genes and VJ gene recombination pairs compared to the age/sex matched control group

TCR repertoires of αβ-T cells in the thymus and PB were constructed for both TRB and TRA from ten MG patients. For the control group, ten age/sex-matched healthy individuals were selected from the TCR repertoires available in a public database [19] or individual studies [16,17] (Supplementary Table 3). From the MG patients, 14,305 to 268,078 productive reads were acquired (Supplementary Table 4) and considered sufficiently high to perform statistical analysis.

The TCR plays a critical role in the T cells' recognition of foreign antigens and initiation of immune responses. TCRs acquire their diversity and specificity from the V(D)J recombination process, and thus the V genes and VJ gene recombination pairs in TCR repertoires are interpreted as the result of a directed immune response to the exposed antigens. Based on this context, the differential usage of V genes and VJ gene recombination pairs in PB of MG patients were investigated and compared to the PB of control group. The

results showed that the usage of certain V genes and VJ gene recombination pairs were significantly upregulated in PB of MG patients for both TRB (Fig. 1) and TRA (Supplementary Fig. 1). When the usage of V genes and VJ gene recombination pairs were compared between the PB and thymi from MG patients, similar usage of VJ gene recombination pairs of both TRB (Fig. 1) and TRA (Supplementary Fig. 2) was observed, which suggests that this particular subset of T cells not only proliferate in the thymus but also circulate in the PB, or migrate from the PB into the thymic tissue, the site of auto-reactive B cell generation [5].

Specifically, the TCRs encoded by the TRBV12-4, TRBV4-3, and TRBV6-6 genes and the TRBV28_TRBJ2 and TRBV29-1_TRBJ2 pairs constituted the TRB repertoires in the PB of MG patients at a significantly higher frequency than the control group. TCRs with TRBV12-4 and TRBV4-3 genes were notably upregulated more than 100 fold in the PB of MG patients, and TCRs with the other V genes and VJ gene recombination pairs (TRBV28_TRBJ2 family, TRBV29-1_TRBJ2 family, and TRBV4-3) were upregulated by approximately 2–10 folds. Therefore, it is possible that these V genes and VJ gene recombination pairs encode TCRs reactive to autoantigens in MG patients (Fig. 1). Similar patterns of preferential V gene usage was also observed in TRA repertoires (TRAV3, TRAV36/DV7, TRAV8-1, TRAV8-2, TRAV8-3, TRAV8-4, and TRAV8-6) in the PB of MG patients (Supplementary Fig. 1). In case of comparing thymi with PB of MG patients, an additional higher usage of TRAV40 in thymi was observed (Supplementary Fig. 2).

3.3. T cells displaying TCRs with longer CDR3 are more abundant in thymi of MG patients

Complementary determining regions (CDRs) are formed during the VJ gene recombination process. In particular, the third complementary determining region (CDR3) of TRB plays a key role in determining TCR specificity [20], and its sequence and length are highly variable compared to other CDRs. In healthy individuals, the distribution of CDR3 lengths show a Gaussian distribution [21]. However, under antigenic stimuli such as exposure to autoantigens in autoimmune disease patients, the distribution can be skewed and autoimmune disease patients were reported to show a biased distribution in CDR3 lengths [22–24]. We compared the distribution of TRB CDR3 lengths between the PB of MG patients and the PB of the control group (Fig. 2a). The distribution of TRB CDR3 lengths in the PB of MG patients and control group did not show significance discrepancy, except the preference for CDR3 lengths of ten amino acid in the PB of MG patients. The comparison between the PB and thymi of MG patients, however, showed biased distribution of TRB CDR3

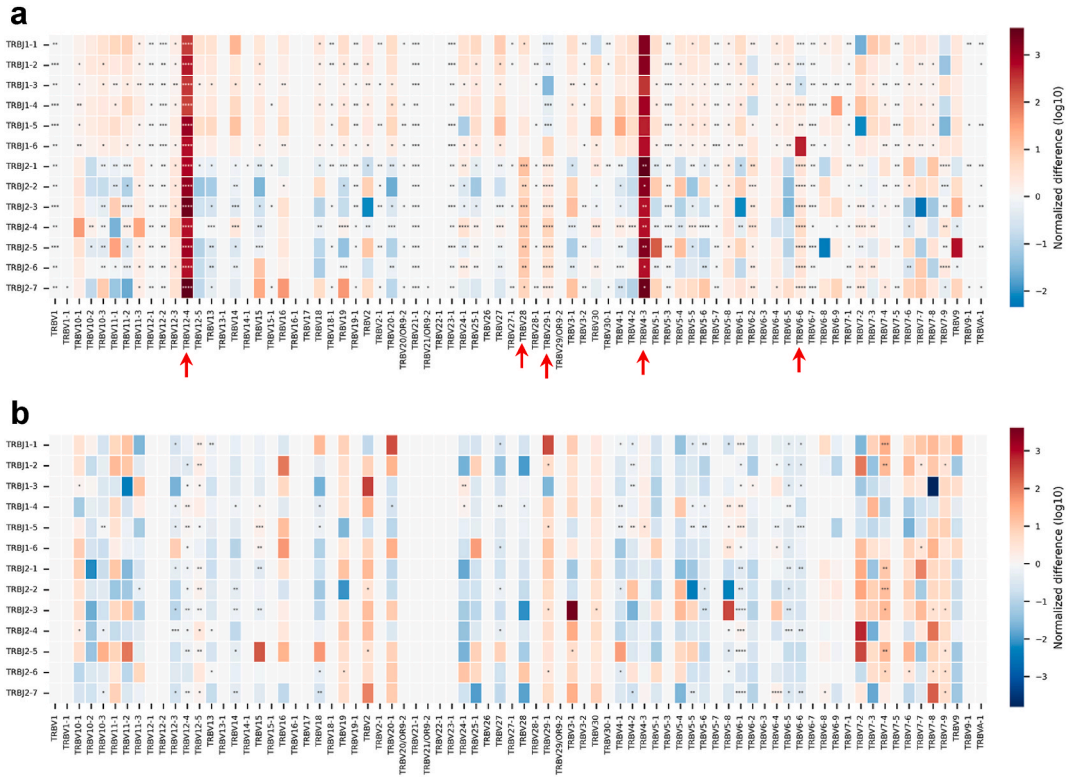

Fig. 1. The usage of VJ gene recombination pairs in the TRB of MG patients. The usage of VJ gene recombination pairs in PB of MG patients was compared with an age/sex-matched healthy control group (n = 10) and thymi of MG patients, respectively. The normalized difference of usage was calculated by subtracting the frequency of each recombination pair in one group from that of the target group, then dividing with the subtracting group's recombination pair frequency. The V genes and VJ gene recombination pairs upregulated in the target group were marked with red arrows. (a) Comparison of the PB of MG patients with the PB of an age/sex-matched healthy control group. (b) Comparison of the thymi with the PB of MG patients. The Mann-Whitney *U* test was applied to test the significance of difference. *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$, ****; $p \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

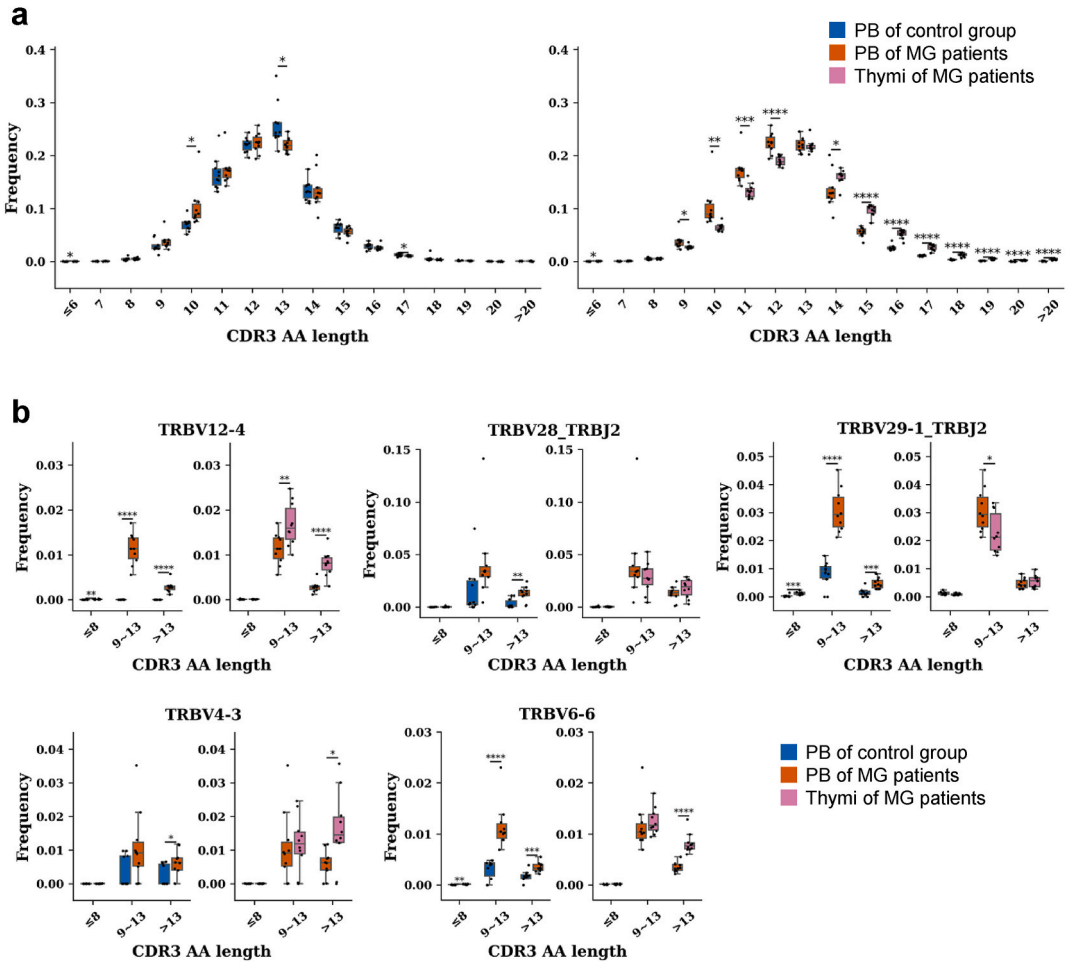

Fig. 2. The distribution of TRB CDR3 lengths in MG patients. The lengths of TRB CDR3 in PB of MG patients were compared with PB of an age/sex-matched healthy control group (left panel of each figure) and thymi of MG patients (right panel of each figure), respectively. (a) The distribution of CDR3 lengths for whole TRB sequences. (b) The distribution of CDR3 lengths for TRB sequences encoded by each significantly upregulated V gene and VJ gene recombination pair in the PB of MG patients. The student t-test was applied to test the significance of the difference. *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$, ****; $p \leq 0.0001$.

lengths toward longer lengths in thymi, and the tendency was statistically significant in CDR3s more than thirteen amino acid residues long (Fig. 2a). Additionally, we confirmed that the TRB encoded by all the upregulated V genes and VJ gene recombination pairs previously identified in the PB of MG patients also favor longer CDR3 lengths with statistical significance compared to the PB of control group (Fig. 2b). When compared thymi of MG patients with PB, longer CDR3 lengths were preferred for TRB encoded by TRBV12-4, TRBV4-3, and TRBV6-6 with statistical significance (Fig. 2b). For TRA repertoires, preference for CDR3 lengths of 14, 16, 17 and 18 amino acid residues was evident in the thymi of MG patients compared to PB (Supplementary Fig. 3a). Among the TCRs that were encoded by the previously identified V genes and VJ gene recombination pairs upregulated in the PB of MG patients, preference for longer CDR3 lengths was confirmed only in TRAV8-1 with statistical significance (Supplementary Fig. 3b). In case of comparing thymi of MG patients with PB, TRAs with longer CDR3 lengths was significantly abundant in TRAV36/DV7 and TRAV40 (Supplementary Fig. 3b).

These results suggest the possibility that T cells displaying TCRs encoded by a specific set of V gene or VJ gene recombination pair and with longer CDR3 lengths play a key role in MG pathology. The described αβ-T cells likely contribute to the capture of auto-antigens, recruitment of autoantigen-reactive B cells, and generation of germinal centers (GCs), resulting in the development of autoantibody-generating B cells in the thymus [5].

3.4. A subset of T cells with TRBs encoded by an upregulated V gene are highly expanded in MG patients

To investigate if MG patients had a biased expansion of αβ-T cell clones, we used the Gini index (0–1) to analyze T cell clonality in the MG group and compare to that of the control group. Higher T cell clonality has been commonly observed in autoimmune disease patients, as the subset of T cells reactive to self-antigens would expand preferentially due to the persistent exposure to autoantigens

[25,26]. However, our analyses showed that the clonality of TRB in MG patients was not increased in the PB compared to the PB of the control group (Fig. 3a). Comparison of the PB and thymi of MG patients also showed no difference in clonality (Fig. 3a). We also checked clonality in the TRBs encoded by the previously identified upregulated V genes and VJ gene recombination pairs (Fig. 3b), and TRBs with longer length-CDR3 (Fig. 3c). TRB sequences encoded by TRBV12-4 showed significantly increased clonality in the PB of MG patients compared to the control group's PB, and no significant difference in clonality was observed between the PB and thymi of MG patients for all upregulated V genes and VJ gene recombination pairs (Fig. 3b). When TRB sequences were sub-divided into groups based on CDR3 length, four groups (8, 13, 15, and 18 amino acid) showed decreased sequence clonality in the PB of MG patients compared to the PB of the control group, and no clonality difference was observed between the PB and thymi of MG patients for all groups (Fig. 3c). The same analysis was applied to TRA sequences, and we identified decreased clonality in the PB of MG patients than the control group's PB regardless of VJ gene usage or CDR3 lengths, and reduction of clonality was more evident in the thymi than the PB of MG patients (Supplementary Fig. 4).

3.5. Stereotypic MG patient-specific TCR clonotypes are identified, and those co-existing in both the PB and thymus show higher frequency compared to those confined to the PB

Disease-related antigens typically lead to the development and expansion of stereotypic TCR sequences which can be commonly found across patients of the same disease. Accordingly, we analyzed the presence of stereotypic TCR clonotypes in MG patients, which we define as identical VJ genes coupled with identical CDR3 amino acid sequences. In either ten MG patients or ten healthy individuals in the control group, we identified the number of subjects with identical TCR clonotypes, and defined this as the sharing degree. The stereotypic clonotypes of TRB and TRA were identified at each sharing degree (Supplementary Figs. 5a and b). In MG patients, the frequencies of stereotypic TRB clonotypes in the PB were significantly higher from the second to seventh sharing degrees compared to the PB of the control group (Supplementary Fig. 5a). As for TRA, the frequency of stereotypic clonotypes were significantly higher from the second to fifth sharing degrees in the MG PB compared to the PB of the control group (Supplementary Fig. 5b). At sharing degrees of six and seven, the frequency of stereotypic TRA clonotypes in the PB of MG patients were significantly lower than that in the PB of the control group.

For the selection of stereotypic TCR clonotypes specific only to MG patients, all stereotypic clonotypes found in MG patients were filtered to exclude clonotypes also found in the datasets of healthy subjects (n = 335 for TRB, n = 22 for TRA). We identified 2,462 stereotypic clonotypes specific to MG patients for TRB, among which 14 clonotypes were present in both the PB and thymi of MG

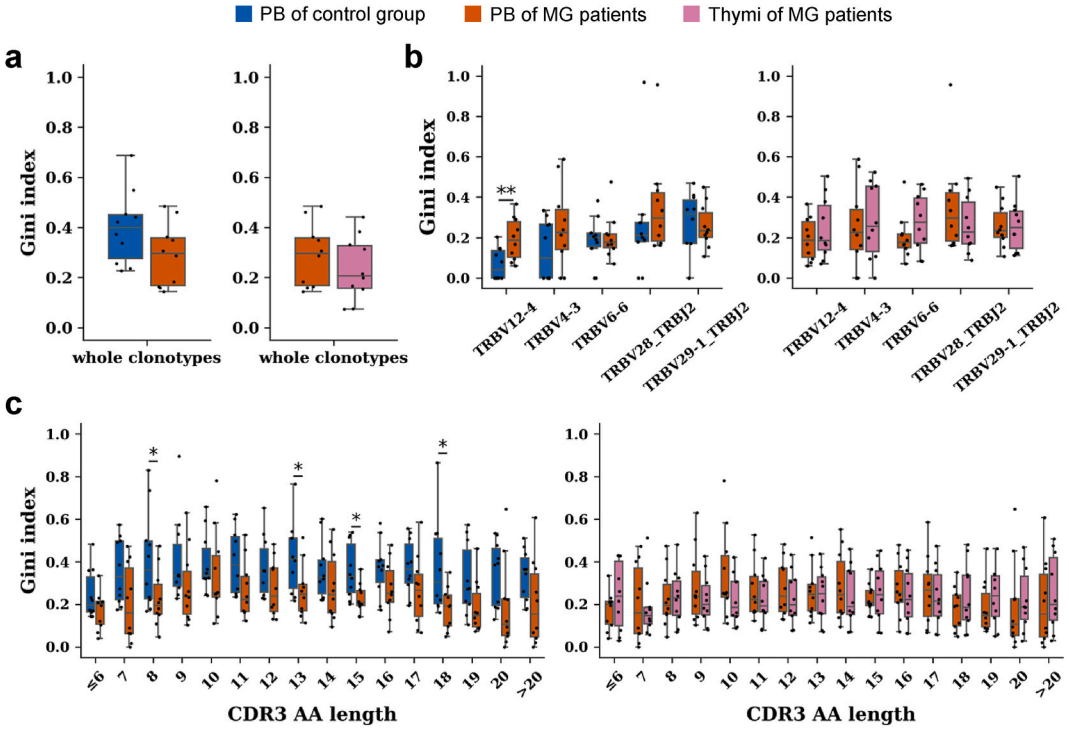

Fig. 3. The clonality of TRB repertoires in MG patients. The Gini index was calculated as a measure of clonality for the TRB repertoires in PB of MG patients and compared with those in PB of an age/sex-matched healthy control group (left panel of each figure) and thymi of MG patients (right panel of each figure), respectively. (a) The clonality of whole TRB sequences. (b) The clonality of TRB sequences encoded by each upregulated V gene and VJ gene recombination pair in the PB of MG patients. (c) The clonality of TRB sequences grouped by lengths of CDR3 amino acid chains. The Mann-Whitney *U* test was applied to test the significance of the difference. *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$, ****; $p \leq 0.0001$.

patients (Fig. 4a, Table 1). For TRA, we identified 45,442 stereotypic clonotypes specific to MG patients.

The relative frequency of stereotypic MG patient-specific clonotypes and whether they are found in only the PB, thymi, or both tissues may determine the fate of pathogenic T cells. We classified the stereotypic MG patient-specific TRB clonotypes based on their localization (found only in PB, only in thymus, or both in PB and thymus), and calculated the frequency of clonotypes in each group for comparison. In the case of clonotypes found in both the PB and thymus, their frequencies were calculated for both. Compared to clonotypes found only in the PB, those found only in the thymus or in both the PB and thymus were identified at a significantly higher frequency (Fig. 4b). This finding suggests that pathogenic T cells are not confined to the thymus, but rather circulate in the PB. For TRA, we identified 4,121 stereotypic MG patient-specific clonotypes co-existing in both PB and thymus (Supplementary Fig. 6a), and the clonotypes found in both the PB and thymus were again identified at a significantly higher average frequency compared to that of PB-confined clonotypes (Supplementary Fig. 6b).

4. Discussions

MG is an antibody-mediated autoimmune disease in which its autoantibodies are known to target AChR proteins at the neuromuscular junctions. The majority of MG patients (~70%) are reported to have anti-AChR antibodies [27], and a large proportion of the patients with anti-AChR antibody-positive MG display abnormal thymus structure [28]. One of the characteristic structural abnormalities is the presence of ectopic germinal centers. The presence of these tertiary lymphoid structures strongly supports that antigen-specific B cells are developed and proliferated in the thymus with the aid of helper T cells [5]. Despite the critical role of helper T cells in the development of B cells, the T cells in MG patients, especially those in thymus, have been less thoroughly studied. Previous research has primarily investigated the changes in the population of T cell subtypes, such as follicular helper T (Tfh) cells, T helper 17 (Th17) cells, or regulatory T (T_{reg}) cells [29–31], and the cytokine profiles of T cell origins [4,30,32–34]. The distribution of TCR CDR3 lengths among MG patients have previously been investigated to find distinct characteristics, but the results were not concordant between studies [35,36], possibly due to the low resolution of past sequencing technologies.

In this study, we aimed to investigate the αβ-T cells in anti-AChR antibody-positive MG patients, and successfully revealed the characteristics of TCR repertoires unique to MG patients compared to the control group. The TRB and TRA of T cells in the PB of MG patients favored the usage of certain V genes or specific VJ gene recombination pairs compared to the PB of the control group with statistical significance. The preferential expansion of T cells with genes encoding for longer CDR3 regions was also confirmed in the TRBs and TRAs of the MG thymi, although to a lesser degree in TRAs. TRB sequences encoded by the TRBV12-4 gene showed significantly increased clonality in the PB of MG patients compared to those in the PB of the control group. When TRB sequences were sub-divided into groups based on theirs CDR3 lengths, four groups showed decreased sequence clonality in the PB of MG patients than the control group's PB. These unique characteristics of TCR repertoires shared by MG patients strongly suggest a directed T cell-mediated immune response driven by the exposure to common autoantigens, as in other autoimmune diseases [25,26].

In autoimmune disease research, TCR repertoire analysis is frequently performed for the differential diagnosis or prognosis of disease activity [9,23,37]. We identified stereotypic MG patient-specific TCR clonotypes present in both the PB and thymi of MG patients, which may potentially be encoded in the pathogenic Tfh cells of MG, as reported previously [30,38]. These clonotypes co-exist in the PB and thymi at a significantly higher frequency than the clonotypes confined to the PB. This observation is in line with our prior knowledge that autoreactive B cells maturate and proliferate in the thymus with the aid of specific subsets of T cells, and the surgical removal of the thymus cannot cure MG [39], as pathogenic T cells are not confined to thymus, but are also present in the PB. Presence of MG specific T cells and their role in the pathogenesis of anti-AChR antibody-positive MG may explain the lack of association between the concentration of anti-AChR antibody and severity of MG [40] as well as the sub-optimal response of B cell

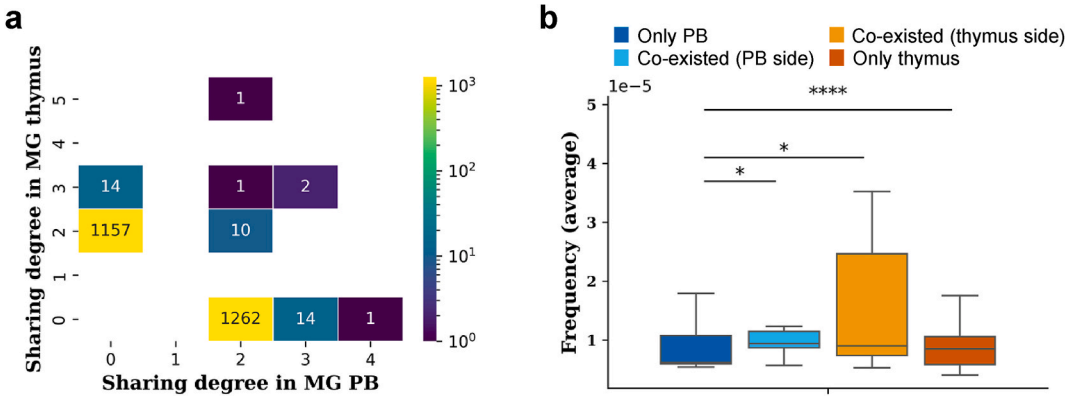

Fig. 4. The distribution of stereotypic MG patient-specific TRB clonotypes. The stereotypic MG patient-specific TRB clonotypes were examined in each sample type (PB, thymus). (a) The number of stereotypic MG patient-specific TRB clonotypes by sharing degree in each sample type. (b) Comparison of the frequency of stereotypic MG patient-specific TRB clonotypes by localization; found only in the PB, both in the PB and thymus, and only in the thymus. The Mann-Whitney *U* test was applied to test the significance of the difference. *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$, ****; $p \leq 0.0001$.

Table 1
Stereotypic MG patient-specific TRB clonotypes found in both the PB and thymus.

V gene	J gene	CDR3	Sample type	Sharing degree	Frequency (x10^{-6})	Detected patients
TRBV12-4	TRBJ2-3	ASSLTDTQY	PB	3	6.1–11.6	AP146, AP154, AP156
			Thymus	3	4.4–12.4	AP77, AP103, AP156
TRBV24-1	TRBJ2-1	ATSDLSGKYNEQF	PB	3	6.1–65.0	AP137, AP139, AP154
			Thymus	3	6.9–9.4	AP77, AP139, AP154
TRBV12-4	TRBJ1-4	ASSLGEKLF	PB	2	6.1–12.3	AP139, AP157
			Thymus	5	3.7–29.6	AP77, AP122, AP146, AP154, AP156
TRBV12-4	TRBJ1-5	ASSLGGSNQPQH	PB	2	6.5–12.2	AP137, AP157
			Thymus	3	3.7–59.2	AP122, AP137, AP154
TRBV12-4	TRBJ2-3	ASSFTDTQY	PB	2	6.3–10.1	AP146, AP159
			Thymus	2	4.7–7.0	AP77, AP146
TRBV12-4	TRBJ2-7	ASSPTSYEQY	PB	2	5.0–6.3	AP146, AP159
			Thymus	2	3.7–66.7	AP154, AP159
TRBV29-1	TRBJ2-5	SVLKQETQY	PB	2	6.3–18.4	AP77, AP146
			Thymus	2	14.0–33.3	AP77, AP159
TRBV12-4	TRBJ2-6	ASSLAGANVLT	PB	2	5.0–18.4	AP77, AP159
			Thymus	2	6.9–12.4	AP103, AP137
TRBV12-4	TRBJ2-2	ASSFSGELF	PB	2	5.0–18.4	AP77, AP159
			Thymus	2	4.7–12.0	AP77, AP137
TRBV10-3	TRBJ2-2	AIRGQGPNTGELF	PB	2	5.8–15.4	AP122, AP156
			Thymus	2	4.7–59.2	AP77, AP122
TRBV12-4	TRBJ2-7	ASSTGGYEQY	PB	2	6.5–12.3	AP137, AP154
			Thymus	2	3.7–7.5	AP154, AP157
TRBV12-4	TRBJ1-1	ASSFVGGGTEAF	PB	2	5.0–35.0	AP103, AP159
			Thymus	2	4.4–12.4	AP103, AP156
TRBV12-4	TRBJ2-2	ASSQGTGELF	PB	2	5.0–17.5	AP103, AP159
			Thymus	2	3.7–6.9	AP139, AP154
TRBV28	TRBJ1-4	ASSFRTPNEKLF	PB	2	12.3–493.5	AP154, AP157
			Thymus	2	3.7–52.6	AP154, AP157

Among all stereotypic MG patient-specific TRB clonotypes, 14 clonotypes co-existed in the PB and thymus. The VJ gene recombination pairs, CDR3 sequences, sharing degrees in each sample type, frequencies, and list of patients are shown.

depletion therapy (i.e., rituximab) in treating patients with anti-AChR antibody-positive MG [41,42].

The limitations of our study are as follows. First, the number of patients was relatively small and thymic histology of the included patients were heterogeneous, consisting of thymoma and thymic hyperplasia. Further studies with larger sample size would be required to assess properly whether the TCR repertoires are different between the patients with thymoma and those with thymic hyperplasia. Second, it is ideal to compare the TCR repertoires between the thymi of MG patients and those of the control group, but only the PB of the control group was used for comparison due to the difficulty of acquiring thymi from healthy donors. Third, TCR repertoires of the control group were mostly derived from Caucasian subjects using different experimental protocols from ours. Therefore, we must consider both the ethnic and technical differences in our analysis of this data. Fourth, we did not characterize the functionality of stereotypic MG patient-specific TCR clonotypes, such as their reactivity to antigenic peptides found in MG patients [3,4]. During sample preparation for NGS, the native pairings of TRA and TRB chains were lost, which limited the characterization of original TCR pairs in pathogenic T cells. Despite these limitations, our study has successfully highlighted the presence of stereotypic MG patient-specific TCR clonotypes that co-exist in both thymus and PB, which strongly suggests a biased T cell-mediated immune response in MG patients.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Yonghee Lee: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Seung Woo Kim:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Eunjae Lee:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Ha Young Shin:** Resources, Data curation. **MinGi Kim:** Investigation, Data curation. **Chang Young Lee:** Supervision. **Byung Jo Park:** Resources, Data curation. **Ha Eun Kim:** Resources, Data curation. **Young Ho Yang:** Resources, Data curation. **Jinny Choi:** Writing – review & editing. **Soyeon Ju:** Methodology. **Jungheum Park:** Methodology. **Namphil Kim:** Data curation. **Jaewon Choi:** Data curation. **Jin Gu Lee:** Supervision, Resources. **Sunghoon Kwon:** Supervision, Funding acquisition. **Junho Chung:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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