Immune Skew of Circulating Follicular Helper T Cells Associates With Myasthenia Gravis Severity

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Abstract

Objective
To clarify functional alterations of follicular helper T cells (Tfh) in myasthenia gravis (MG) because Tfh play important roles in helping B cells generate antibody-producing cells.

Methods
A total of 24 immunotherapy-naive patients with anti–acetylcholine receptor (AchR) antibody–positive MG and 18 age-matched healthy subjects (HS) were enrolled. Samples from 6 patients were available for posttreatment analysis. Subsets of circulating Tfh (cTfh) and B cells were identified by flow cytometry analysis of surface molecules. Cytokine production by isolated cTfh subsets from 5 patients with MG and 5 HS was measured in vitro. Analysis was performed to examine the correlation between the frequency of cTfh subsets and that of plasmablasts and between cTfh subsets and the quantitative MG score.

Results
cTfh increased with elevated expression of inducible T-cell costimulator (ICOS) in patients with MG. cTfh shifted to Th2 and Th17 over Th1 in MG. ICOS\textsuperscript{high} cTfh produced significantly higher levels of interleukin (IL)-21, IL-4, and IL-17A than ICOS\textsuperscript{low} cTfh only in patients with MG. The frequency of cTfh within CD4 T cells was more closely associated with disease severity than the serum anti-AchR antibody titer and frequency of plasmablasts within B cells. Abnormalities of cTfh were improved after immunotherapy in parallel with clinical improvement.

Conclusions
Alternation of cTfh is a key feature in the development of MG and may become a biomarker for disease severity and therapeutic efficacy.

Classification of Evidence
This study provides Class II evidence that the level of cTfh is associated with disease severity in patients with MG.
Myasthenia gravis (MG) is an antibody-mediated autoimmune disease targeting neuromuscular junctions, characterized by fluctuating muscle weakness with easy fatigability. About 80% of patients with MG are positive for anti-acetylcholine receptor (AChR) antibody. However, the serum concentration of anti-AChR antibody is not always associated with the clinical severity. Hence, it is difficult to clarify the clinical severity based solely on the anti-AChR antibody titer.

Accumulating evidence has shown that follicular helper T cells (Tfh), chemokine (C-X-C motif) receptor 5 (CXCR5)-expressing CD4 T cells present in secondary lymph organs, play a vital role in B-cell maturation and antibody production. Tfh preferentially express inducible T-cell costimulator (ICOS), which plays crucial roles in promoting B-cell maturation and antibody production by interacting with the ICOS ligand on B cells.

Although Tfh and antibody-producing cells such as plasma cells in secondary lymph nodes are a focus of interest in antibody-mediated autoimmune diseases, these cells are difficult to access. Previous studies treated CXCR5+ CD4 T cells in peripheral blood (PB) as circulating Tfh (cTfh) and regarded plasmablasts as counterparts of plasma cells in PB. These studies were based on the premise that CXCR5+ CD4 T cells in PB largely share functional properties with Tfh in secondary lymph nodes and show an increase of cTfh in human antibody-mediated autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren syndrome, and neuromyelitis optica.

Evidence shows that Tfh in PB and secondary lymph nodes can be divided into Tfh1, Tfh2, and Tfh17-like total helper T cells and that Tfh2 and Tfh17 more strongly induce antibody production. Among other cytokines, interleukin (IL)-21 is preferentially secreted by Tfh. We analyzed cTfh1, cTfh2, and cTfh17 shifts in patients with MG and studied cytokine production including IL-21 by cTfh in vitro.

In addition, we analyzed cTfh1, cTfh2, and cTfh17 shifts in patients with MG and studied cytokine production including IL-21 by cTfh in vitro. We analyzed the association of the cTfh phenotype with disease severity in patients with MG and its phenotypic change before and after treatment.

Methods
Patients
We included 24 immunotherapy-naive hospitalized patients with MG with anti-AChR antibody attending Kyoto Prefectural University of Medicine, Kyoto University, or Kansai Medical University Medical Center (from March 2017 to November 2018) and 18 age-matched healthy subjects (HS) (table). Six of the 24 immunotherapy-naive patients who could be followed up were analyzed 2–4 weeks after the initiation of all forms of immunotherapy (table).

The clinical data including quantitative MG (QMG) score were evaluated by neurologists different from those performing flow cytometric analysis, and the serum anti-AChR antibody level (cutoff: 0.2 nmol/L) was commercially examined by radioimmunoassay (SRL, Inc., Japan) at the point of blood sample collection.

Standard Protocol Approvals, Registrations, and Patient Consents
This study was approved by the medical ethics committee of Kyoto Prefectural University of Medicine (RBMR-G-140-1). All subjects provided written informed consent.

Sample Collection and Flow Cytometric Analysis
PB mononuclear cells (PBMCs) were isolated from fresh blood samples with Lymphocyte Separation Medium 1077 (Wako, Japan) according to the manufacturer’s instructions. PBMCs were stained with antibodies from BioLegend (San Diego, CA) unless otherwise stated. CD4 T cells were identified with anti-human CD3 allophycocyanin (APC)-cyanine (Cy) 7 (clone SK7) and anti-human CD4 peridinin chlorophyll protein (PerCP)-Cy5.5 (clone OKT4), anti-human CXCR5 Alexa Fluor 488 (clone J252D4), anti-human ICOS phycoerythrin (PE) (clone C398.4A), anti-human CXCR3 Brilliant Violet 510 (clone 1C6), anti-human C-C receptor (CCR) 6 PE-Cy7 (clone G034E3), and control isotype-matched antibodies. To identify B-cell subsets, we used anti-human CD19 PE-Cy7 (clone HIB19), anti-human CD27 APC (clone M-T271) (BD Biosciences, Franklin Lakes, NJ), anti-human CD38 PerCP-Cy5.5 (clone HIT2), and anti-human CD138 pacific blue (clone M115). The data were acquired using a FACS Canto II flow cytometer (BD Biosciences). Although T cells were analyzed in all of the enrolled
subjects, B-cell analysis was performed for 23 patients with MG and 12 HS.

**Measurement of Cytokine Production by cTfh**

For analysis of cytokine production, we used PBMCs derived from 5 patients with MG and the same number of HS. PBMCs were stained as indicated above and sorted into CXCR5-positive and -negative CD4 T cells or ICOS high expressing (ICOS high) and low expressing (ICOS low) CXCR5+ CD4 T cells by Cell Sorter SH800 (Sony, Japan). Sorted CD4 T cells were cultured at a density of 2 × 10⁵/mL for 48 hours in AIM-V (Thermo Fisher Scientific, Japan) in 96-well U-bottom plates. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin cell stimulation (Sigma-Aldrich Co., LLC, St. Louis, MO) during cell culture. The concentration of cytokines was measured in the supernatant with BD CBA (Thermo Fisher Scientific, Japan) and the data were analyzed using FCAP Array v.3.0 software (BD Biosciences).

**Statistical Analysis**

Data are presented as the mean ± SD. Statistical analysis was conducted with the Mann-Whitney U test for analysis of unpaired data. Spearman rank correlation analysis was used for correlation analysis, and the paired t test was used to assess the difference between before and after treatment. All data sets were analyzed using JMP 13 (SAS Institute Inc., Cary, NC). A p value of <0.05 was considered significant.

**Data Availability**

The data sets generated and analyzed during the current study are available from the corresponding author on reasonable request.

### Results

**Frequency and Phenotypic Analysis of cTfh Within CD4 T Cells**

First, we analyzed cTfh, namely CXCR5+ CD3⁺ CD4⁺ cells, and their expression of ICOS (figure 1, A–C). The frequency of cTfh was determined as a percentage of CD4 T cells. The frequency of cTfh significantly increased in patients with MG compared with HS (9.0% in patients with MG vs 5.8% in HS, p = 0.0015) (figure 1, A and B). The frequency of ICOS⁺ cTfh significantly increased in patients with MG compared with HS (4.0% in patients with MG vs 0.9% in HS, p < 0.0001) (figure 1, A and C). Also, the frequency of

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**Table Patient Background**

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### Cases with follow-up samples

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Abbreviations: AchR = acetylcholine receptor; EOMG = early-onset MG; LOMG = late-onset MG; CS = corticosteroid; IVIg = IV immunoglobulin; MG = myasthenia gravis; post = posttreatment; pre = pretreatment; QMG score = quantitative MG score.
ICOS\textsuperscript{high} cells within cTfh was significantly higher in patients with MG than in HS (39.2% in patients with MG vs 11.6% in HS, \( p < 0.001 \)) (figure 1D).

Similarly to total CD4 T cells, cTfh cell subsets included 3 distinct subsets: cTfh1, cTfh2, and cTfh17.\textsuperscript{6,9,15} In this study, we regarded CXCR3+ CCR6- CXCR5+ CD4 T cells as cTfh1, CXCR3- CCR6- CXCR5+ CD4 T cells as cTfh2, and CXCR3- CCR6+ CXCR5+ CD4 T cells as cTfh17 based on previous reports.\textsuperscript{6,9,15} The frequency of cTfh2 and cTfh17 within CD4 T cells was significantly higher in patients with MG than in HS (5.7% in patients with MG vs 4.2% in HS, \( p = \)).

**Figure 1** cTfh Frequency and ICOS Expression on cTfh and cTfh Subsets

(A) Representative flow cytometry dot plots of CXCR5 expression on CD4 T cells from patients with MG and HS (left) and representative flow cytometry dot plots showing expression of CXCR5 and ICOS (right). (B) Frequency of cTfh within CD4 T cells shown for comparison between patients with MG and HS. Frequency of ICOS\textsuperscript{high} cTfh within CD4 T cells (C) and within cTfh (D) for comparison between patients with MG and HS. Frequency of cTfh1 (CXCR3+ CCR6- cTfh), cTfh2 (CXCR3- CCR6- cTfh), and cTfh17 (CXCR3- CCR6+ cTfh) within CD4 T cells (E) and cTfh1, cTfh2, and cTfh17 subsets within cTfh (F) was also analyzed. Data are indicated as the mean ± SEM. cTfh = circulating follicular helper T cell; HS = healthy subjects; ICOS = inducible T-cell costimulator; MG = myasthenia gravis; PBMC = peripheral blood mononuclear cell.
We subsequently analyzed cytokine production by ICOShigh cTfh in patients with MG vs HS, 20.1 pg/mL in patients with MG vs 8.6 pg/mL in HS, IL-4 and IL-17A production by cTfh was significantly higher in patients with MG vs 14.7 pg/mL in HS, respectively (figure 1F). ICOShigh cTfh within CD4 T cells was not associated with that of plasmablasts (p = 0.9) (figure e-1E). The frequency of plasmablasts within lymphocytes was also not significantly correlated with the frequency of cTfh1 or that of cTfh2 within CD4 T cells (p = 0.38 and 0.16, respectively) (figure e-1, F and G). On the other hand, the frequency of plasmablasts showed a significant correlation with the cTfh17 frequency within CD4 T cells (R² = 0.22, p = 0.023) (figure e-1H).

Cytokine Production by ICOShigh and ICOSlow cTfh

We examined cytokine production by cTfh from patients with MG and HS in response to PMA and ionomycin. ICOShigh cTfh and ICOSlow cTfh were sorted as shown in figure 2A. There was no significant difference in IFN-γ production between patients with MG and HS (84.2 pg/mL in patients with MG vs 154.5 pg/mL in HS, p = 0.83) (figure 2B). In contrast, IL-4 and IL-17A production by cTfh was significantly higher in patients with MG than in HS (37.0 pg/mL in patients with MG vs 2.7 pg/mL in HS) and ICOSlow cTfh ( ICOShigh cTfh = 0.012, respectively). The production of IL-17A by cTfh within CD4 T cells was more significantly correlated with the QMG score than ICOSlow cTfh (ICOShigh cTfh: R² = 0.31, p = 0.0038, and ICOSlow cTfh: R² = 0.17, p = 0.039) (figure 3, A and B).

Association of Disease Severity With Phenotype of cTfh, Anti-AchR Antibody Titer, and Plasmablasts

We examined the association of disease severity with the cTfh phenotype. The disease severity was significantly correlated with the frequency of cTfh within CD4 T cells (R² = 0.35, p = 0.005) (figure 3A). Furthermore, ICOShigh cTfh within CD4 T cells was more significantly correlated with the QMG score than ICOSlow cTfh (ICOShigh cTfh: R² = 0.31, p = 0.0038, and ICOSlow cTfh: R² = 0.17, p = 0.039) (figure 3A and B). Although the frequency of cTfh1 and cTfh17 within CD4 T cells showed no significant correlation with the QMG score (figure e-2, A and C, links.lww.com/NXI/A376), that of cTfh2 within CD4 T cells showed a significant correlation with the score (R² = 0.22, p = 0.02) (figure e-2B).

There was no correlation between the serum anti-AchR antibody titer or the frequency of plasmablasts and QMG score (figure e-2, D and E, links.lww.com/NXI/A376).

Association of Clinical Characteristics With Phenotype of cTfh

We compared the phenotype of cTfh between patients with MG with and without thymoma. There was no significant difference in the cTfh frequency within CD4 T cells between patients with MG with and without thymoma (10.1% and 8.1%, respectively, p = 0.98) (figure e-3A). The frequency of ICOShigh cTfh cells within cTfh did not show any significant difference between patients in MG with or without thymoma (38.0% and 40.4% in patients with or without thymoma, respectively, p = 0.77) (figure e-3B). There was no clear difference in the cTfh1, cTfh2, or cTfh17 frequency within cTfh between patients with MG with or without thymoma (figure e-3C).

Next, we compared the phenotype of cTfh between patients with early-onset MG (EOMG) (aged <50 years) and patients with late-onset MG (LOMG) (aged ≥50 years). The frequency of cTfh within CD4 T cells was significantly higher in patients with EOMG than patients with LOMG (10.8% in patients with EOMG vs 7.3% in patients with LOMG, p = 0.019) (figure e-3D, links.lww.com/NXI/A377). This difference should be carefully interpreted because patients with EOMG tended to exhibit a higher QMG score (10.2 in patients with EOMG vs 6.6 in patients with LOMG). On the contrary, there was no significant difference in the ICOShigh cell frequency within cTfh between these 2 groups (p = 0.66) (figure e-3E). Furthermore, there was no significant difference...
in the cTfh1, cTfh2, or cTfh17 frequency within cTfh between patients with EOMG and LOMG (figure e-3F).

cTfh, Plasmablasts, and Anti-AchR Antibody Level After Immunotherapy

We analyzed the cTfh phenotype before and after starting immunotherapy. All 6 patients showed an improved QMG score after immunotherapy (p = 0.002) (figure 3D). The frequency of cTfh and ICOS^{high}cTfh within CD4 T cells was significantly reduced after immunotherapy (p = 0.04 and 0.02, respectively) (figure 3, E and F).

The frequency of cTfh1 and cTfh2 within CD4 T cells decreased after treatment (p = 0.1 and 0.04, respectively) (figure e-4, A and B, links.lww.com/NXI/A378). The change in the cTfh17 frequency was the most significant (p = 0.009) (figure e-4C). The frequency of plasmablasts within B cells was reduced in 5 patients after treatment, but without significance (p = 0.16) (figure e-4D). The serum anti-AchR antibody level tended to decrease (figure e-4E).

Discussion

In this study, we demonstrated an increase of cTfh in patients with MG, consistent with 2 previous reports. An increased frequency of Tfh was also previously observed in the thymus and thymoma associated with MG. When we further analyzed cTfh subsets based on the expression of chemokine receptors, cTfh2 and cTfh17 dominance over cTfh1 was observed. Of interest, the frequency of cTfh2 and cTfh17 within CD4 T cells reduced after immunotherapy. The increase of cTfh17 in MG was also reported previously, and our observation on cTfh2 dominance is not fully consistent with previous reports on MG. Although the patients we analyzed showed acute worsening after onset, the other studies enrolled patients not receiving immunotherapy for more than 2 years. An increased frequency of Th2 was correlated with higher disease activity of SLE. Taken together, different activities between patients examined may have led to different results. The production of IL-4 and IL-17A by cTfh was significantly greater in patients with MG,
which supports the Th2 and Th17 dominance observed by surface molecule analysis in them.

In this study, we also found that ICOS\textsuperscript{high} cTfh increased, and IL-21 production was upregulated in this cTfh subset of patients with MG. The interaction between ICOS on cTfh and its ligand on B cells is essential for B-cell development and antibody production.\textsuperscript{11} ICOS knockout mice were reported to be resistant to clinical experimental autoimmune MG development with lower levels of serum AchR-specific immunoglobulins.\textsuperscript{25} In our analysis, cTfh exhibited significantly higher expression of ICOS in patients with MG compared with HS, a finding in agreement with a recent report.\textsuperscript{26}

IL-21 is a key cytokine of cTfh\textsuperscript{17,18} and promotes B-cell differentiation and antibody production.\textsuperscript{27-29} A study of ICOS-deficient mice reported that effector T cells showed an impaired ability to produce IL-21 and IL-4, and IgG production was also reduced.\textsuperscript{25} In our study, IL-21 production by cTfh was markedly elevated in patients with MG, IL-21 specifically induces IgG1 and IgG3, but not IgG2 nor IgG4.\textsuperscript{30} The preferential production of IL-21 may play an important role in producing anti-AchR antibody that exclusively belongs to IgG1 and IgG3 subclasses.\textsuperscript{31} Of interest, this upregulated IL-21 production was only observed in ICOS\textsuperscript{high} cTfh in patients with MG. Of note, the production of IL-4 and IL-17A by ICOS\textsuperscript{high} cTfh from patients with MG increased in parallel with IL-21 (figure 2C). The elevation of IL-21–, IL-4–, and IL-17A–producing cell frequencies within CD4 T cells in MG was recently reported, supporting our results.\textsuperscript{26} These observations showed that cTfh, especially qualitative and quantitative changes of ICOS\textsuperscript{high} cTfh, are essential for the pathogenic cytokine condition in MG. Although ICOS\textsuperscript{high} Tfh were reported to promote germinal center B cells to produce IgG compared with ICOS\textsuperscript{low} Tfh,\textsuperscript{32} the functional difference between ICOS\textsuperscript{high} and ICOS\textsuperscript{low} Tfh remains elusive. We reported on enhanced cytokine production by ICOS\textsuperscript{high} cTfh in a human pathologic condition.

IL-21 plays a crucial role in the differentiation of Th17 in the presence of transforming growth factor β\textsuperscript{33,34} and that of Tfh themselves in the presence of IL-17.\textsuperscript{35} It was also reported that IL-21 could promote the Th2 response\textsuperscript{36,37} and suppress development of the Th1 response.\textsuperscript{38} Hence, it is likely that the upregulation of IL-21 and ICOS high expression on Tfh is an upstream functional skew to a Th2- and Th17-biased condition and preparation for a condition where pathogenic antibody is produced.

An increase of plasmablasts was reported in several autoimmune disorders including MG.\textsuperscript{17,39} However, the frequency of plasmablasts itself did not reach significance in our study. This suggests that the skew of cTfh is more sensitive to detect the pathogenic condition compared with that of plasmablasts.

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**Figure 3** Association of Disease Severity With Phenotype of cTfh and Tfh Phenotypic Change Before and After Treatment

The QMG score was correlated with the frequency of the following subsets within CD4 T cells: (A) cTfh, (B) ICOS\textsuperscript{high} cTfh, (C) ICOS\textsuperscript{low} cTfh. In (A), the circle indicates EOMG, and the triangle indicates LOMG. The QMG score and frequency of cTfh subsets within either CD4 T cells or cTfh were analyzed before and after immunotherapy. (D) QMG score. (E) Frequency of cTfh and (F) ICOS\textsuperscript{high} cTfh within CD4 T cells. cTfh = circulating follicular helper T cell; ICOS = inducible T-cell costimulator; MG = myasthenia gravis; post = after immunotherapy; pre = before immunotherapy; QMG score = quantitative MG score.
Consistent with previous observations, the anti-AchR antibody titer was not correlated with the disease severity in this study. Anti-AchR antibodies in patients with MG are comprised of antibodies that exhibit different biological activities. The functional activities of antibodies differ among patients, which might explain, at least in part, why titers of antibodies do not correlate with the disease severity.

In contrast and most importantly, quantitative changes in cTfh were correlated with the disease severity in patients with MG, at least before treatment. The presence or absence of thymoma did not influence the frequency of cTfh, ICOS expression, or Th2 or Th17 dominance within cTfh. In addition, elevation of the cTfh frequency in patients with EOMG compared with patients with LOMG may be attributed to differences in the disease severity between the 2 groups. These facts have raised the possibility that analysis of cTfh can be used as an immunologic marker in MG. This was also supported by the observation that the cTfh frequency decreased in parallel with improvement of the clinical severity.

This study had some limitations. First, this was a small study. We were unable to analyze ICOShigh cTfh in patients with MG with exacerbation. We could not conclude whether ICOShigh cTfh is a marker to predict improvement or exacerbation of clinical symptoms because we did not follow up patients with MG at several predetermined points. Hence, a further prospective long-term study is needed to confirm the utility of ICOShigh cTfh as an activity marker of MG in clinical settings.

In conclusion, we revealed that ICOShigh cTfh are elevated and cTfh shift to Th2 and Th17 in MG. Functionally activated Tfh are key features in the immunopathology of MG. Of clinical importance is the fact that the frequency of cTfh was correlated with disease severity in patients with MG.

Acknowledgment
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Disclosure

Publication History

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<tr>
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<td>Assisted and oversaw experimental design and data interpretation and supervised the process of writing the manuscript</td>
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References


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# Immune Skew of Circulating Follicular Helper T Cells Associates With Myasthenia Gravis Severity

Shinji Ashida, Hirofumi Ochi, Mio Hamatani, et al.
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