RESEARCH ARTICLE

In-depth peripheral CD4⁺ T profile correlates with myasthenic crisis

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Abstract

Objective: Myasthenia gravis (MG) is an autoimmune disease caused by autoantibodies against neuromuscular junctions. Myasthenic crisis (MC) represents the most severe state of MG with high in-hospital mortality. We aimed to identify immune signatures using in-depth profiling in MC, and to assess the correlations between immune biomarkers with clinical severity longitudinally. **Methods:** We studied 181 participants including 57 healthy controls, 96 patients with MG who never experienced crisis and 28 MC patients from December 2018 through June 2020. Follow-up visits occurred prospectively from crisis to 6 months off-mechanical ventilation. The frequencies of 20 CD4⁺ T subpopulations and 18 serum cytokines were associated with clinical scores using correlations and principal component analysis. **Results:** Patients in crisis exhibited a proinflammatory CD4⁺T response with elevated Th1 ($P = 0.026$), and Th17 cells ($P = 0.032$); decreased T follicular helper 2 (Tfh2) cells ($P < 0.001$), Tnaive in Tfh cells ($P < 0.001$), ICOS⁻Tfh cells ($P = 0.017$), and T central memory in Tfh ($P = 0.022$) compared with controls, and increased frequencies of Tregs ($P = 0.026$) and Tfh17 ($P = 0.045$) compared with non-crisis MG. Cytokine cascade was identified in crisis including the ones associated with Th1 (IL-1β/2/12p70/18/27/IFN-γ/TNF-α), Th2 (IL-4/5/13), Th17 (IL-6/17A/21/22/23/GM-CSF), Th9 (IL-9), and Treg (IL-10). Longitudinally, seven immune biomarkers including Tregs, IL-2/4/17A/IFN-γ/TNF-α/GM-CSF had significant correlations with MG-activities of daily living score. **Interpretation:** Vigorous inflammatory CD4⁺ T signatures were identified in MC and are associated with clinical severity. Future research is needed to explore its potential candidacy for therapeutic intervention and predicting impending crisis.

Introduction

Myasthenia gravis (MG) is an autoimmune disease that is characterized by fatigable weakness in extraocular, bulbar, limb, and axial muscles with a prevalence of 40–180 per million people worldwide.[1,2] The disease generally begins with ocular symptom and extends to other muscles in 80% of patients.[3,4] Rapid worsening dysphagia and respiratory failure indicate myasthenic crisis (MC), which is a potentially life-threatening complication requiring ventilatory support. MC occurs in approximately 15–20% of MG patients in their lifetime.[5] The incidence of post-

thymectomy MC is 11.5–18.2% and the in-hospital mortality rate is 10–12%.[6–10] Thus patients in crisis represent the most serious clinical condition of MG and potentially undergo a vigorous autoimmune process.[11] Given the devastating nature of the MC state, prognostic immune biomarkers would be especially valuable for predicting disease severity and providing insights for the development of therapies, as well as monitoring therapy efficacy.

Immune dysregulation in MG including autoantibodies production, imbalance among CD4$^+$ T subsets such as Th1, Th2, Th17, and T regulatory (Treg) cells, and the involvement of CD8$^+$ cells and antigen-presenting cells.[12] Autoantibodies against the acetylcholine receptor (AChR), muscle-specific kinase (MuSK), and lipoprotein-related protein 4 are well established as sensitive and specific diagnostic biomarkers.[13–15] However, there is no correlation between antibody titer and the disease activity.

Notably, peripheral CD4$^+$ T lymphocytes play important roles in the immunopathogenesis process in MG, as evidenced by the presence of autoreactive CD4$^+$ T subsets with elevated IL-17, IFN-r, and GM-CSF,[16–18] as well as upregulated follicular T helper (Tfh) cells.[19,20] In particular, the immune profile of patients in crisis remained poorly understood. Thus, comprehensive and dynamic profiling of peripheral CD4$^+$ T lymphocyte and cytokines in MC may facilitate the understanding of the immunological process and provide promising candidates for disease biomarkers and targets for therapeutic development.

Herein, we hypothesized that peripheral CD4$^+$ T immune responses have correlations with the disease severity of MG, with activated CD4$^+$ T cells and proinflammatory cytokines dominating the process of MC. We also hypothesized that associations would be detected between inflammatory biomarkers and the clinical severity after MC. In this study, peripheral blood mononuclear cells and serum were obtained from MG patients in or after the crisis longitudinally, and correlations were assessed among the peripheral CD4$^+$ T and cytokine profiles with clinical MG scores.

Patients and Methods

Study subjects

A total of 181 participants were recruited in our study. Twenty-eight consecutive patients in crisis (also classified as Myasthenia Gravis Foundation of America Class V, MGFA V) with mechanical ventilation (MV) were prospectively recruited from five neurological intensive care units (NICUs) or neurologically associated interdisciplinary intensive care units (ICUs) in North, West and General campus of Huashan Hospital Fudan University, Shanghai. Multiple visits for clinical evaluations and sampling were

achieved in crisis (at the first 3 days with ventilatory support, $n = 28$), at 1 week ($n = 24$), 1 month ($n = 18$), 2 months ($n = 13$), 3 months ($n = 13$) and 6 months off-ventilation ($n = 15$). A cohort of 96 generalized MG patients with the MGFA Class II–IV who had never had a crisis was included from the neuromuscular clinic or neurological department of Huashan Hospital for comparative analysis. All participants were aged 18 years or older and recruited from 1 December 2018, through 4 June 2020. All participants met the inclusion criteria as follows: (1) clinical history and signs of fluctuating weakness and fatigability; (2) seropositivity for AChR/MuSK antibodies; and (3) if negative for the tested antibodies, positive repetitive nerve stimulation (RNS) at low frequency (2–5 Hz) is required.[21,22] Participants were excluded if the RNS had over 100% increase after high-frequency stimulation or R2 repeats, to differentiate from lambert-Eaton syndrome and congenital MG. The MG participants were excluded if they had the concurrence of fever when sampling. Healthy controls, enrolled through the hospital, were excluded if they had a previous diagnosis of neuroimmune diseases or systemic autoimmune disorders or any fever or sickness at the time of blood sampling.

Clinical assessment

Each participant had been evaluated by MG-activities of daily living (MG-ADL) scale and MG Quality of Life 15 (QoL-15) questionnaire. MGFA-quantitative MG test (MGFA-QMG), and MG manual muscle test (MMT) were evaluated by experienced MG specialists.

Laboratory testing and data acquisition

Serum antibody testing

Serum antibodies were measured against AChR and MuSK using Enzyme-linked immunosorbent assays (ELISA, Euroimmun, Lübeck, Germany). The serums from participants were obtained and firstly sent for anti-AChR antibody testing. If the result was negative, the anti-MuSK antibody was subsequently tested. The optical density was read at 450 nm within 30 min of adding the stop solution. The result was presented as antibody titers using four Parameter-Logistics fitting method to calculate the concentration. The cut-off threshold for anti-AChR and MuSK antibodies are 0.50 nmol/L and 0.4 U/mL.

Flow cytometry immunophenotyping of cell subsets

Blood samples were drawn from each participant into tubes containing EDTA and sent immediately for flow

cytometry analysis. A 300 μL aliquot of whole blood was incubated with a cocktail of titrated, monoclonal, fluorochrome-labeled antibodies in the dark for 20 min at 20°C. Then whole blood was lysed twice with red blood cell lysing buffer (TIANGEN, Beijing, China) by centrifugation at 350g for 5 min at 20°C, followed by two washes with phosphate buffer saline and re-suspension in fluorescence activating cell sorter buffer. Cell phenotyping was performed using Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by Flowjo VX Software (Flowjo, LLC (BD life sciences, Franklin Lakes, NJ, US)). $CD4^+$ T-cell profiling was performed using a cell-surface protocol according to previous studies.[23–25] Briefly, we first gated $CD3^+CD4^+$ T cells and $CD3^+CD4^-$ T cells, and at least 100,000 events were acquired per sample in the lymphocyte gate. Then the frequency of $CD4^+$ T subsets was identified as follows: Tnaive: $CD27^+CD45RA^+$; T central memory (TCM): $CD27^+CD45RA^-$; T effect memory (TEM): $CD27^-CD45RA^-$; T effect memory-ra (TEMRA): $CD27^-CD45RA^+$; Th1: $CXCR3^+CCR6^-$; Th2: $CXCR3^-CCR6^-$; Th9: $CCR4^-CCR6^+$; Th17: $CXCR3^-CCR6^+$; Th17/1: $CXCR3^+CCR6^+$; Tfh: $CXCR5^+$; $ICOS^+$Tfh: $ICOS^+CXCR5^+$; $ICOS^-$Tfh: $ICOS^-CXCR5^+$; Tfh1: $CXCR5^+CXCR3^+CCR6^-$; Tfh2: $CXCR5^+CXCR3^-CCR6^-$; Tfh17: $CXCR5^+CXCR3^-CCR6^+$; Tnaive in Tfh: $CXCR5^+CD27^-CD45RA^+$; Tfh central memory (TCM in Tfh): $CXCR5^+CD27^+CD45RA^-$; Tfh effect memory (TEM in Tfh): $CXCR5^+CD27^-CD45RA^-$; Tfh effect memory-ra (TEMRA in Tfh): $CXCR5^+CD27^-CD45RA^+$; Treg: $CD25^{hi}CD127^{dim}$.

Serum cytokines analysis

Serum was isolated by centrifugation at 1690 **g** for 15 min at 20°C, subsequently followed by the preservation at −80°C. $CD4^+$ T-cell-related cytokines were measured using a ProcartaPlex™ Multiplex panel (Thermo Fisher, EPX180-12165-901) according to the product instructions including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IFN-γ, TNF-α, and GM-CSF. The cytokine concentrations were calculated using the standard curve generated by the five-parameter logistic regression method. In samples with undetectable cytokines, the values of the detection limit were used for analysis.

Statistical analysis

Descriptive statistics were presented as means ± SD for continuous variables; frequency and proportions for categorical variables. For lymphocyte subsets, the proportions less than 0.05% were not taken into statistical analysis.

Multiple subgroup comparisons were analyzed using one-way ANOVA, Kruskal–Wallis, or Cochran-Mantel-Haensel test. A correlation matrix was generated to analyze pairwise correlations among all variables derived from the MC cohort using Spearman's method. A multivariate regression model aiming to adjust confounders including gender and age was used to measure the effect of each variable on the MG-ADL score. Principle component analysis (PCA) was used to explore the inter-relationships among variables derived from the MC cohort in terms of patients at distinct phases. All procedures with multiple comparisons were adjusted by the Hochberg method. We considered a two-tailed adjusted $P < 0.05$ as statistically significant. Data analysis was done using Stata 14.0, GraphPad Prism 7.0 software, (GraphPad Software, LLC, San Diego, CA, USA) and R software 4.0.2.

Results

Demographics and clinical characteristics

One hundred and eighty-one participants took part in the study. Of these, 124 patients had MG (72 women [58.1%] and 52 men [41.9%]; mean [SD] age, 48.6 [16.0] years) and 57 participants were healthy controls (28 women [49.1%] and 29 men [50.9%], mean [SD] age, 48.9 [14.1] years). MG patients included 28 patients experienced MC (MC cohort), and 96 non-crisis MG with no experience of manifest crisis (Non-MC cohort) according to the 2016 AAN international consensus guidance (Table 1).[26] There was no gender difference in patients recruited and 25 cases (89.3%) in the MC cohort provided multiple clinical samples. The median age at sampling was not significantly different among MC cohort (mean [SD] age, 52.5 [16.4] years), Non-MC cohort (mean [SD] age, 47.3 [15.3] years), and healthy controls (mean [SD] age, 48.9 [14.1] years).

The clinical classifications at first sampling in the MC cohort were MGFA class V (28/28, 100%). The Non-MC cohort was mainly comprised of MGFA class II (65/96, 67.7%) and class III (26/96, 27.1%) at sampling. A high prevalence of thymoma concurrence was apparent in the MC cohort (19/28, 67.9%) compared with that in the Non-MC group (25/96, 26.0%, $P < 0.001$). For the preceding events for the crisis, 11 patients developed post-operative crisis, six with precipitating upper respiratory infection, one with diarrhea, three with over-exercise, two with rapid drug escalation, and five patients with unknown triggers. MC cohort had a significantly higher prevalence of immunosuppressants treatment (28/28, 100%) in comparison to the Non-MC cohort (24/96, 25%, $P < 0.001$). All participants in crisis had been treated with glucocorticoids and intravenous immunoglobulin.

Table 1. Population demographics and clinical findings of the study population.

Characteristic	MG (n = 124)		Healthy controls (n = 57)
	MC cohort (n = 28)	Non-crisis MG cohort (n = 96)	
Age, mean (SD), y	52.5 (16.4)	47.3 (15.3)	48.9 (14.1)
Sex, No. (%)			
Male	14 (50.0)	38 (39.6)	29 (50.88)
Female	14 (50.0)	58 (60.42)	28 (49.12)
MGFA Class at first sampling No. (%)			
II	0 (0)	65 (67.7)	NA
III	0 (0)	26 (27.1)	NA
IV	0 (0)	5 (5.2)	NA
V	28 (100)	0 (0)	NA
Immunosuppressants therapy, No. (%)	28 (100)***	35 (36.5)	NA
Thymoma concurrence, No. (%)			
Thymoma associated	19 (67.9)***	25 (26.0)	NA
No thymoma	9 (32.1)	71 (74.0)	NA
Preceding events for crisis, No. (%)			
Post-operative myasthenic crisis	11 (39.3)	NA	NA
Upper respiratory infection	6 (21.4)	NA	NA
Diarrhea	1 (3.6)	NA	NA
Over-exercise	3 (10.7)	NA	NA
Rapid drug escalation	2 (7.1)	NA	NA
Unknown triggers	5 (17.9)	NA	NA
Antibody, No. (%), mean (SD),nmol/L			
Anti-AChR Ab	25 (89.3), 9.9 (5.5)	90 (93.8), 7.9 (6.2)	NA
Anti-MuSK Ab	2 (7.1), 2.1 (2.6)	3 (3.1), 4.3 (6.6)	NA
Seronegative	1 (3.6)	3 (3.1)	NA
Immunotherapy at sampling, No. (%)			
Glucocorticoids	28 (100)***	24 (25)	NA
High dose	10 (35.7)**	0 (0)	NA
Low dose with gradual escalation	18 (64.3)**	24 (100)	NA
Immunoglobulin	28 (100)***	5 (13.8)	NA
Azathioprine	1 (3.6)	14 (14.6)	NA
Tacrolimus	5 (17.9)	21 (21.9)	NA
Mycophenolate mofetil	0 (0)	1 (1.0)	NA
Rituximab	0 (0)	5 (5.2)	NA
No immunotherapy	0 (0)***	72 (75)	NA

MG, myasthenia gravis; MC, myasthenic crisis; MGFA, Myasthenia Gravis Foundation of America; AChR, acetylcholine receptor; MuSK, muscle-specific kinase.
**$P < 0.01$.
***$P < 0.001$.

Regarding the laboratory data, in the MC cohort, 25 patients were anti-AChR antibody-positive (89.3%, mean [SD] nmol/L, 9.9 [5.5] nmol/L), two patients were anti-MUSK antibody-positive, and one was seronegative. In the Non-MC cohort, 90 patients were anti-AChR antibody-positive (93.8%, mean [SD] nmol/L, 7.9 [6.2] nmol/L), three patients were anti-MUSK antibody positive, and three were seronegative.

Immune T-cell kinetics and cytokines in MC

$CD3^+CD4^+$ and $CD3^+CD4^-$ T cells were identified using flow cytometry analysis in peripheral blood samples (Fig. 1). In comparison to the Non-MC cohort (n = 96) and healthy controls (n = 57), all lymphocytes significantly decreased in MC cohort (n = 28) (0.21×10^6 vs. 0.45×10^6/mL, $P < 0.001$ and 0.21×10^6 vs. 0.59×10^6/mL, $P < 0.001$, respectively). The relative number of $CD3^+CD4^+$ cells were significantly decreased in crisis in comparison to non-crisis MG and healthy controls (0.05×10^6 vs. 0.15×10^6/mL, $P < 0.001$ and 0.05×10^6 vs. 0.23×10^6/mL, $P < 0.001$). Besides, the relative number of $CD3^+CD4^-$ cells were decreased in crisis in comparison to non-crisis MG and healthy controls (0.06×10^6 vs. 0.13×10^6/mL, $P < 0.001$ and 0.06×10^6 vs. 0.19×10^6/mL, $P = 0.0013$). By further gating on $CD3^+CD4^+$ T cells, we performed an in-depth immune profiling of 20 subpopulations (Table 2). Patients in crisis exhibited an activated inflammatory state with increased proportions of Th1 cells (11.76% vs.
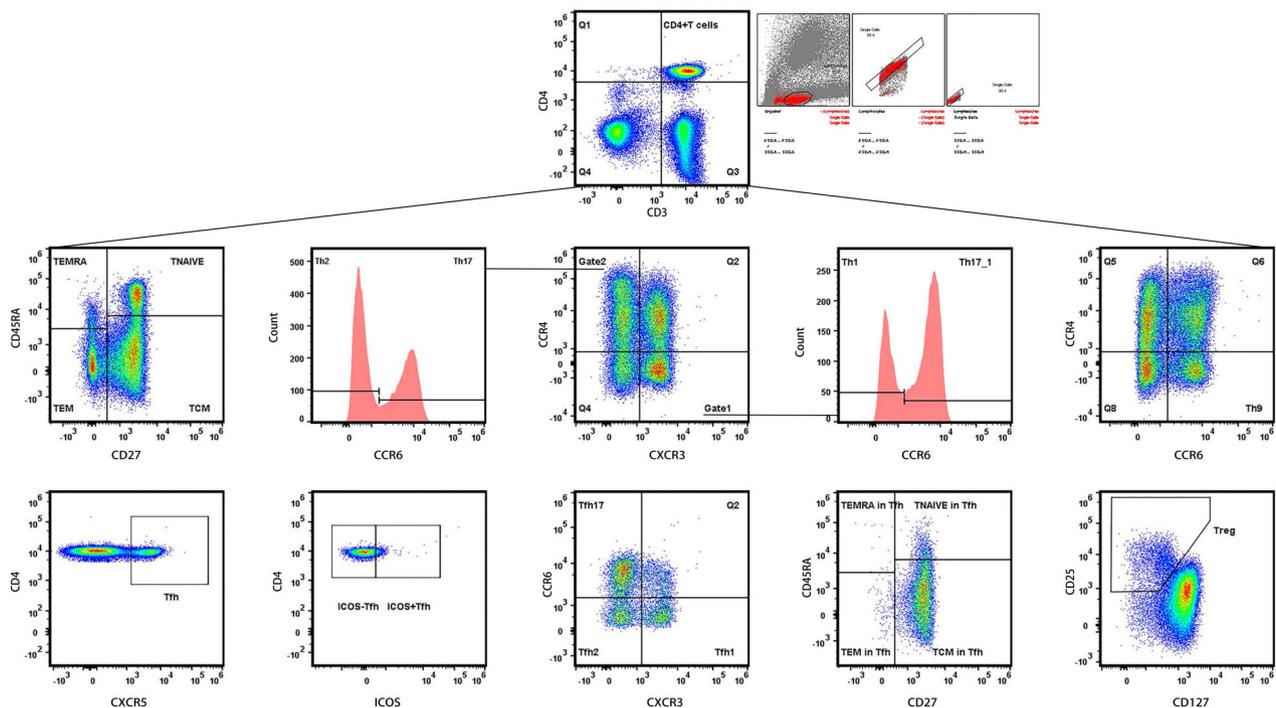
Figure 1. Gating Strategy for peripheral CD4+T subpopulations. CD27 and CD45RA were used to gate naïve T, T central memory (TCM), T effect memory (TEM), and T effect memory-ra (TEMRA). CXCR3, CCR4, and CCR6 were used to gate Th1, Th2, Th9, Th17, and Th17/1. CXCR5 was used to gate Tfh; CD25 and CD127 were used to gate Treg.

8.51%, $P = 0.026$), and Th17 cells (16.17% vs. 12.83%, $P = 0.032$); decreased Tfh2 cells (3.56% vs. 6.35%, $P < 0.001$), Tnaive in Tfh cells (1.45% vs. 3.01%, $P < 0.001$), ICOS⁻Tfh cells (11.68% vs. 15.69%, $P = 0.017$), and TCM in Tfh cells (12.86% vs. 16.77%, $P = 0.022$) compared with healthy controls. In particular, elevated frequencies of Treg (8.21% vs. 6.94%, $P = 0.026$) and Tfh17 cells (6.91% vs. 5.31%, $P = 0.045$) were revealed in patients with crisis compared with those in non-crisis. There were no significant differences among other CD3⁺CD4⁺ T subpopulations.

Cytokine multiplex immunoassay revealed pan-elevation of clustered cytokines including the ones associated with Th1 (IL-1β, IL-2, IL-12p70, IL-18, IL-27, IFN-γ, TNF-α), Th2 (IL-4, IL-5, IL-13), Th17 (IL-6, IL-17A, IL-21, IL-22, IL-23, GM-CSF), Treg (IL-10), and Th9 (IL-9) in MG patients with crisis compared with that in controls and MG in non-crisis (Table 2). In crisis, cytokines were increased over twofold than non-crisis, including Th1-associated molecules IL-1β (3.79 vs. 1.63 pg/mL, 95% CI: 0.66–3.66 pg/mL, $P < 0.001$), IL-2 (25.42 vs. 11.73 pg/mL, 95% CI: 3.59–23.8 pg/mL, $P < 0.001$) and IL-27 (6.82 vs. 1.83 pg/mL, 95% CI: 2.34–7.64 pg/mL, $P < 0.001$), Th2-associated cytokines IL-4 (22.9 vs. 9.38 pg/mL, 95% CI: 4.47–22.55 pg/mL, $P < 0.001$), IL-5 (3.02 vs. 1.2 pg/mL, 95% CI: 0.63–3.01 pg/mL, $P < 0.001$), and IL-13 (2.14 vs. 0.78 pg/mL, 95% CI: 0.5–2.21 pg/mL, $P < 0.001$), Th17-

associated molecules IL-6 (7.54 vs. 1.5 pg/mL, 95% CI: 3.12–8.96 pg/mL, $P < 0.001$), IL-17A (7.26 vs. 2.47 pg/mL, 95% CI: 1.95–7.63 pg/mL, $P < 0.001$) and GM-CSF (34.49 vs. 14.62 pg/mL, 95% CI: 6.23–33.51 pg/mL, $P < 0.001$), as well as Tregs-associated IL-10 (1.56 vs. 0.57 pg/mL, 95% CI: 0.38–1.61 pg/mL, $P < 0.001$). In comparison to the cytokines measured in healthy controls, we detected eight cytokines with over twofold increase in crisis including Th1-associated IL-18 (14.96 vs. 7.39 pg/mL, 95% CI: 1.3–13.84 pg/mL, $P = 0.0041$), IL-27 (6.82 vs. 2.2 pg/mL, 95% CI: 1.9–7.35 pg/mL, $P = 0.011$), and TNF-α (3.17 vs. 1.59 pg/mL, 95% CI: 0.25–2.91 pg/mL, $P = 0.0083$), Th2-associated IL-5 (3.02 vs. 1.35 pg/mL, 95% CI: 0.42–2.92 pg/mL, $P = 0.016$) and IL-13 (2.14 vs. 0.95 pg/mL, 95% CI: 0.29–2.09 pg/mL, $P = 0.033$), Th17-associated IL-6 (7.54 vs. 1.18 pg/mL, 95% CI: 3.43–9.28 pg/mL, $P < 0.001$), IL-17A (7.26 vs. 2.85 pg/mL, 95% CI: 1.46–7.36 pg/mL, $P < 0.001$), and Tregs-associated IL-10 (1.56 vs. 0.44 pg/mL, 95% CI: 0.5–1.74 pg/mL, $P < 0.001$).

Dynamic peripheral CD4⁺ T profile from MC to remission

Longitudinal follow-up of peripheral CD4⁺ T profile and cytokines in patients after the crisis revealed a dynamic and variable alteration (Fig. 2). Consistently decreased frequencies of CD4⁺ T cells were revealed at multiple

Table 2. Peripheral CD4^{+} T lymphocytes and cytokines of study participants.

T subtype (% in CD4+T)	Cytokines (pg/mL)	Crisis (mean)	Non-crisis (mean)	Healthy controls (mean)	Crisis versus non-crisis			Crisis versus healthy controls			Non-crisis versus healthy controls		
					Difference (95% CI)	P sig	P adj	Difference (95% CI)	P sig	P adj	Difference (95% CI)	P sig	P adj
Th1		11.76	10.31	8.51	-3.37 to 6.27	–	0.21	-1.63 to 8.14	>	0.026	-1.22 to 4.82	–	0.12
	IL-1β	3.79	1.63	2.15	0.66 to 3.66	>>>	<0.001	0.01 to 3.26	>>	0.0078	-1.33 to 0.29	–	0.3
	IL-2	25.42	11.73	15.05	3.59 to 23.8	>>>	<0.001	-0.63 to 21.37	>>	0.0063	-9 to 2.35	–	0.51
	IL-12p70	0.34	0.2	0.36	0.01 to 0.29	>>	0.0019	-0.19 to 0.16	–	0.38	-0.29 to -0.04	<	0.018
	IL-18	14.96	8.63	7.39	0.28 to 12.4	>>	0.0061	1.3 to 13.84	>>	0.0041	-1.9 to 4.36	–	0.83
	IL-27	6.82	1.83	2.2	2.34 to 7.64	>>>	<0.001	1.9 to 7.35	>	0.011	-1.21 to 0.47	–	0.063
	IFN-γ	9.91	6.45	10.07	-0.6 to 7.53	>>	0.0011	-5.26 to 4.94	–	0.34	-7.3 to 0.05	<<	0.0036
	TNF-α	3.17	2.27	1.59	-0.42 to 2.21	–	0.065	0.25 to 2.91	>>	0.0083	-0.05 to 1.41	–	0.12
Th2		18.7	21.22	18.5	-10.64 to 5.61	–	0.23	-8.22 to 8.63	–	0.99	-3.69 to 9.13	–	0.13
	IL-4	22.9	9.38	14.09	4.47 to 22.55	>>>	<0.001	-1.18 to 18.8	–	0.058	-9.86 to 0.46	<	0.023
	IL-5	3.02	1.2	1.35	0.63 to 3.01	>>>	<0.001	0.42 to 2.92	>	0.016	-0.67 to 0.38	–	0.25
	IL-13	2.14	0.78	0.95	0.5 to 2.21	>>>	<0.001	0.29 to 2.09	>	0.033	-0.53 to 0.2	–	0.14
Th17		16.17	13.87	12.83	-4.3 to 8.9	–	0.14	-3.51 to 10.2	>	0.032	-3.29 to 5.38	–	0.27
	IL-6	7.54	1.5	1.18	3.12 to 8.96	>>>	<0.001	3.43 to 9.28	>>>	<0.001	-0.2 to 0.83	–	0.5
	IL-17A	7.26	2.47	2.85	1.95 to 7.63	>>>	<0.001	1.46 to 7.36	>>>	<0.001	-1.48 to 0.71	–	0.85
	IL-21	3.12	3.18	2.76	-1.45 to 1.32	<	0.017	-1.15 to 1.88	–	0.3	-0.74 to 1.58	–	0.15
	IL-22	19.67	10.12	16.27	1.66 to 17.43	>>>	<0.001	-5.93 to 12.73	–	0.18	-12.05 to -0.24	<<	0.0054
	IL-23	3.02	1.56	2.85	0.25 to 2.67	>	0.019	-1.33 to 1.67	–	0.78	-2.31 to -0.28	<<	0.0087
	GM-CSF	34.49	14.62	21.77	6.23 to 33.51	>>>	<0.001	-2.41 to 27.87	>	0.04	-15.13 to 0.85	–	0.16
Treg		8.21	6.94	7.06	-2.13 to 4.66	>	0.026	-2.46 to 4.75	–	0.058	-2.43 to 2.2	–	0.54
	IL-10	1.56	0.57	0.44	0.38 to 1.61	>>>	<0.001	0.5 to 1.74	>>>	<0.001	-0.06 to 0.32	–	0.66
Th9		12.67	9.7	10.22	-2.1 to 8.05	–	0.27	-2.94 to 7.84	–	0.62	-3.81 to 2.76	–	0.53
	IL-9	5.83	2.23	3.24	1.3 to 5.89	>>>	<0.001	0.1 to 5.08	–	0.13	-2.2 to 0.19	<<	0.0052
Tnaive		22.63	28.5	28.28	-16.14 to 4.4	–	0.073	-16.95 to 5.65	–	0.068	-9.13 to 9.57	–	0.97
TCM		49.52	50.14	49.41	-21.82 to 20.59	–	0.76	-22.61 to 22.84	–	0.5	-15.64 to 17.1	–	0.49
TEM		24.43	16.53	16.87	-1.89 to 17.69	–	0.065	-2.66 to 17.78	–	0.065	-5.85 to 5.18	–	0.96
TEMRA		3.41	4.83	5.45	-3.03 to 0.19	–	0.64	-3.96 to -0.12	–	0.87	-2.34 to 1.11	–	0.46
Th17/1		7.44	6.56	5.33	-2.17 to 3.94	–	0.75	-0.97 to 5.2	–	0.084	-0.68 to 3.13	∧	0.025
Tfh		15.37	16.42	18.11	-7.62 to 5.52	–	0.48	-10.12 to 4.64	–	0.076	-7.42 to 4.05	–	0.13
ICOS + Tfh		2.95	3.16	4.13	-1.61 to 1.2	–	0.97	-3.13 to 0.77	–	0.066	-2.62 to 0.68	<	0.048
ICOS-Tfh		11.68	11.81	15.69	-5.62 to 5.36	–	0.69	-11.52 to 3.51	<	0.017	-10.11 to 2.36	<<<	<0.001
Tfh1		2.83	3.78	3.24	-2.23 to 0.35	–	0.097	-1.75 to 0.94	–	0.55	-0.59 to 1.67	–	0.23
Tfh2		3.56	4.63	6.35	-2.68 to 0.55	–	0.074	-4.89 to -0.67	<<<	<0.001	-3.61 to 0.17	<<<	<0.001
Tfh17		6.91	5.31	6.76	-1.17 to 4.37	>	0.045	-2.95 to 3.26	–	0.85	-3.5 to 0.61	<<	0.002
Tnaive in Tfh		1.45	2.07	3.01	-1.37 to 0.13	–	0.27	-2.82 to -0.31	<<<	<0.001	-2.13 to 0.24	<<<	<0.001
TCM in Tfh		12.86	12.93	16.77	-6.11 to 5.96	–	0.89	-12.04 to 4.22	<	0.022	-10.53 to 2.86	<<	0.0029
TEM in Tfh		0.44	0.12	0.19	0.14 to 0.51	–	0.084	0.05 to 0.45	–	0.35	-0.15 to 0	<<	0.0023
TEMRA in Tfh		≤0.05	≤0.05	0.051	0.000 to 0.043	–	0.39	-0.030 to 0.025	–	0.011	-0.04 to -0.01	–	<0.001

P sig, *P* value significant; *P* adj, *P* adjusted; "–", no significant difference; ">" and "<", *P* < 0.05; ">>" and "<<", *P* < 0.01; ">>>" and "<<<", *P* < 0.001; TCM, T central memory; TEM, T effect memory. We considered the *P* value of lymphocyte subsets as non-significant due to the small proportion (<0.05%).
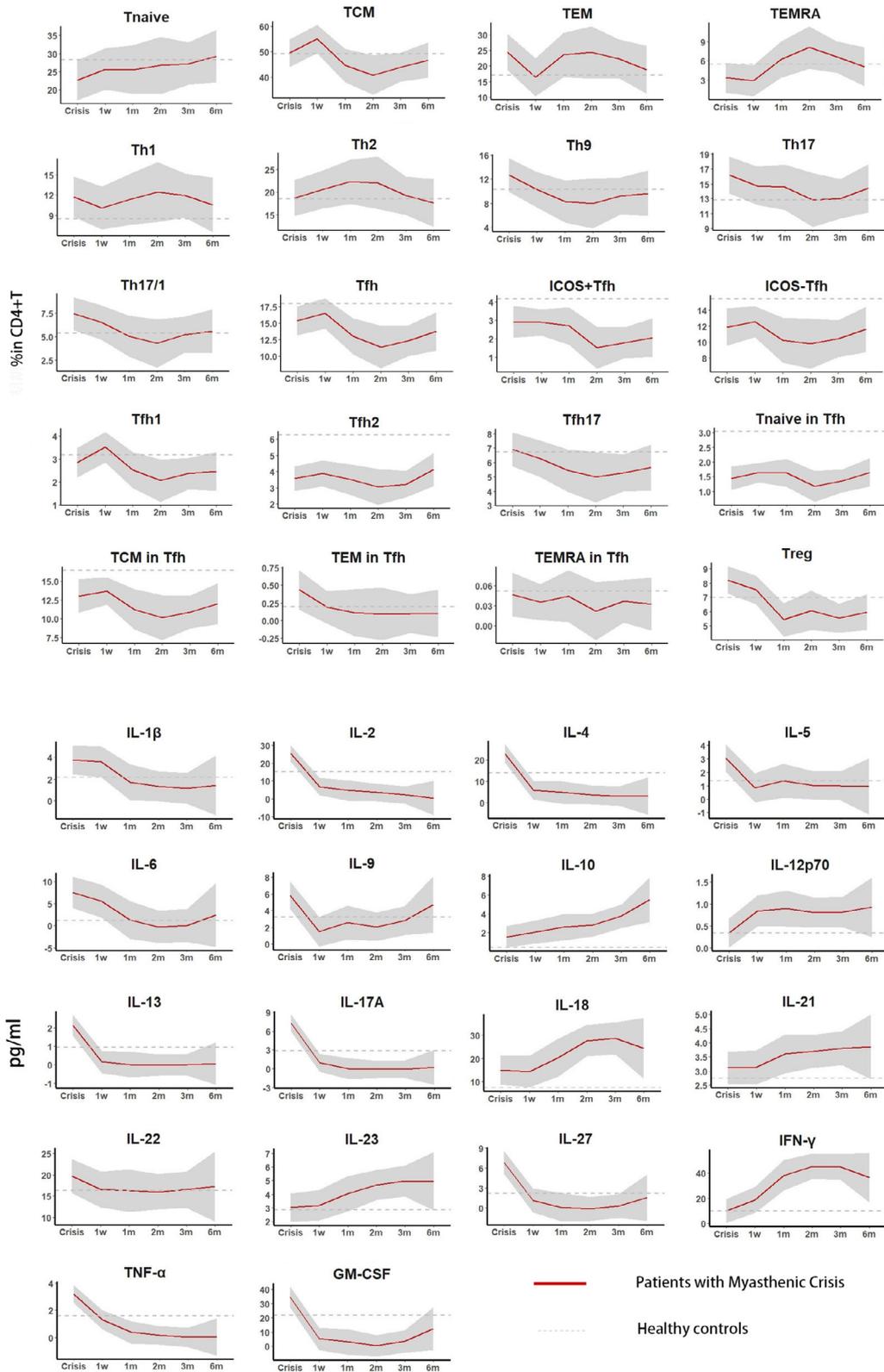
Figure 2. Longitudinal analysis of peripheral CD4$^+$T and cytokine profile in MC cohort. We measured peripheral immune response in crisis ($n = 28$), at 1 week ($n = 24$)/1 month ($n = 18$)/2 months ($n = 13$)/3 months ($n = 13$)/6 months off-ventilation ($n = 15$) in patients with MC. MC, myasthenic crisis.

Table 3. Paired comparison between peripheral CD4$^+$ T profile from patients in crisis state and that at 6 months off-ventilation.

T subsets (% in CD4$^+$T)	Cytokines (pg/mL)	Crisis (mean)	6 months off-ventilation (mean)	Crisis versus 6 months off-ventilation		
				Difference (95% CI)	P sig	P adj
Th1		10.68	10.59	−5.17 to 5.35	−	0.972
	IL-1β	4.90	1.42	−0.97 to 7.92	−	0.096
	IL-2	36.25	0.01	21.91 to 50.56	>>	0.002
	IL-12p70	0.48	1.02	−1.59 to 0.50	−	0.221
	IL-18	18.62	24.06	−14.55 to 3.67	−	0.173
	IL-27	6.32	1.71	−3.63 to 12.85	−	0.195
	IFN-γ	13.66	38.62	−47.84 to −2.08	<	0.039
	TNF-α	2.92	0.01	−0.663 to 6.49	−	0.087
Th2		16.95	17.81	−5.97 to 4.23	−	0.721
	IL-4	35.91	3.77	23.15 to 41.13	>>	0.001
	IL-5	4.14	0.82	−1.19 to 7.84	−	0.111
	IL-13	2.22	0.06	−0.38 to 3.93	>	0.028
Th17		17.45	14.36	−1.6 to 7.76	−	0.178
	IL-6	5.77	2.42	−5.24 to 11.93	−	0.34
	IL-17A	10.68	0.17	7.21 to 13.80	>>	0.001
	IL-21	2.93	3.94	−2.10 to 0.07	−	0.06
	IL-22	17.89	16.63	−7.00 to 9.55	−	0.69
	IL-23	2.65	4.91	−6.50 to 1.98	−	0.21
	GM-CSF	53.24	24.06	−9.87 to 87.47	−	0.091
Treg		7.52	5.91	0.33 to 2.89	>	0.017
	IL-10	1.96	6.07	−14.46 to 6.23	−	0.331
Th9		14.32	9.53	0.16 to 9.75	−	0.057
	IL-9	7.52	5.56	−9.79 to 13.71	−	0.668
Tnaive		23.4	30.56	−18.66 to 4.34	−	0.202
TCM		49.46	46.53	−9.42 to 15.29	−	0.617
TEM		24.22	17.88	−6.28 to 18.97	−	0.297
TEMRA		2.91	5.03	−4.61 to 0.37	−	0.089
Th17/1		7.06	5.47	0.79 to 3.97	−	0.174
Tfh		16.19	13.65	0.77 to 5.84	−	0.122
ICOS + Tfh		2.40	2.30	−1.97 to 2.15	−	0.92
ICOS-Tfh		12.72	12.44	−3.15 to 3.73	−	0.852
Tfh1		2.35	2.41	−0.88 to 0.77	−	0.891
Tfh2		3.98	4.13	−1.5 to 1.2	−	0.815
Tfh17		8.18	5.63	−0.73 to 4.35	>>	0.009
Tnaive in Tfh		1.45	1.79	−1.39 to 0.73	−	0.488
TCM in Tfh		12.89	12.99	−5.42 to 5.24	−	0.969
TEM in Tfh		0.85	0.06	−0.92 to 2.50	−	0.316
TEMRA in Tfh		0.11	0.0047	−0.044 to 0.249	−	0.144

P sig, *P* value significant; *P* adj: *P* adjusted; "−", no significant difference; ">" and "<", $P < 0.05$; ">>" and "<<", $P < 0.01$; ">>>" and "<<<", $P < 0.001$; TCM, T central memory; TEM, T effect memory. We considered the *P* value of lymphocyte subsets as non-significant due to the small proportion (<0.05%).

visits. An activated TEM and Th1 subpopulations were identified continuously from the time of crisis to 6 months off-ventilation. Additionally, we found continually decreased frequencies of Tfh, Tfh2, Tnaive in Tfh, TCM in Tfh, TEM in Tfh, TEMRA in Tfh, and Treg cells. Multiple measurements of cytokines revealed a concordant trend among the ones associated with Th1 (IL-2, TNF-α), Th2 (IL-2, IL-4, IL-5, IL-6, IL-13, IL-27), Th9 (IL-9), and Th17 (IL-6, IL-17A, GM-CSF), with significant elevation at crisis followed by a marked decrement within 1-week off-ventilation.

Paired comparisons between the CD4$^+$ T profile in crisis and that in 6 months off-ventilation revealed significant reductions in frequencies of Tregs ($P = 0.017$) and Tfh17 ($P = 0.009$), as well as in concentrations of serum IL-2 ($P = 0.002$), IL-4 ($P = 0.001$), IL-13 ($P = 0.028$), and IL-17A ($P = 0.001$). Additionally, an elevation in serum IFN-γ concentration was also noted ($P = 0.0039$) in 6 months off-ventilation (Table 3).

Correlations of CD4$^+$ T subpopulations and cytokines with clinical MG scores

We assessed the correlations among all clinical scores and biomarkers derived from MC cohort. It revealed significant three correlations of IFN-γ ($r = -0.529$, $P < 0.001$),
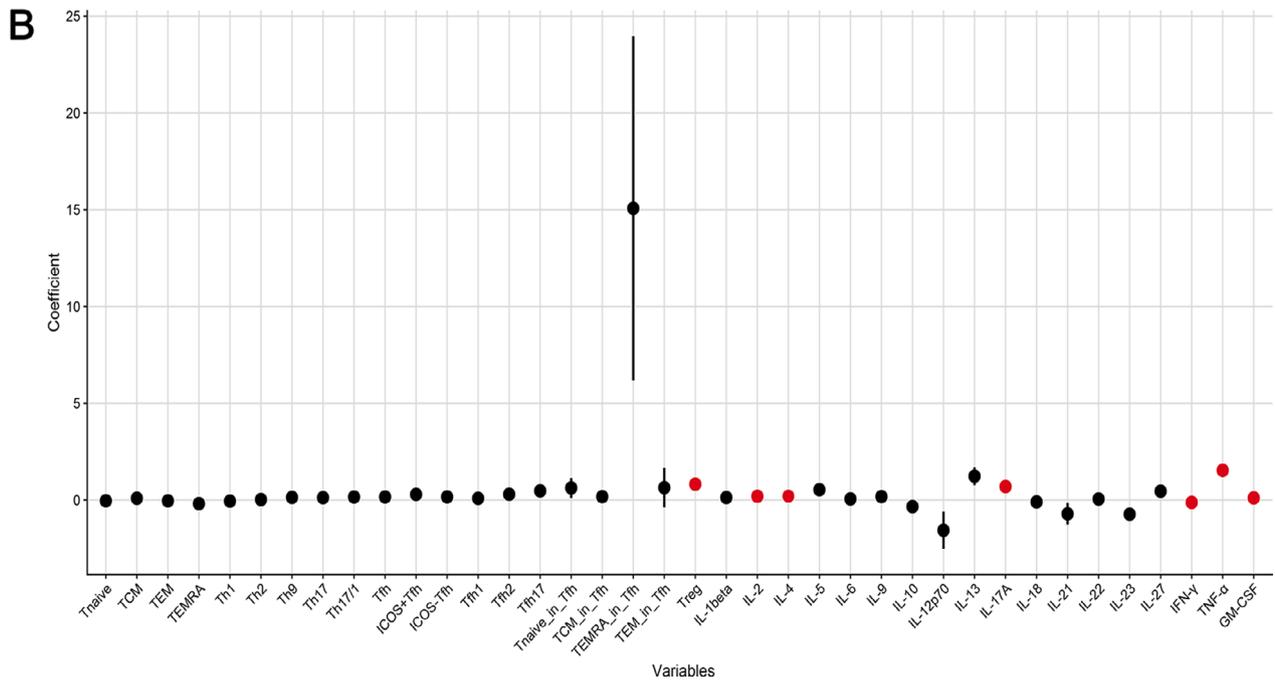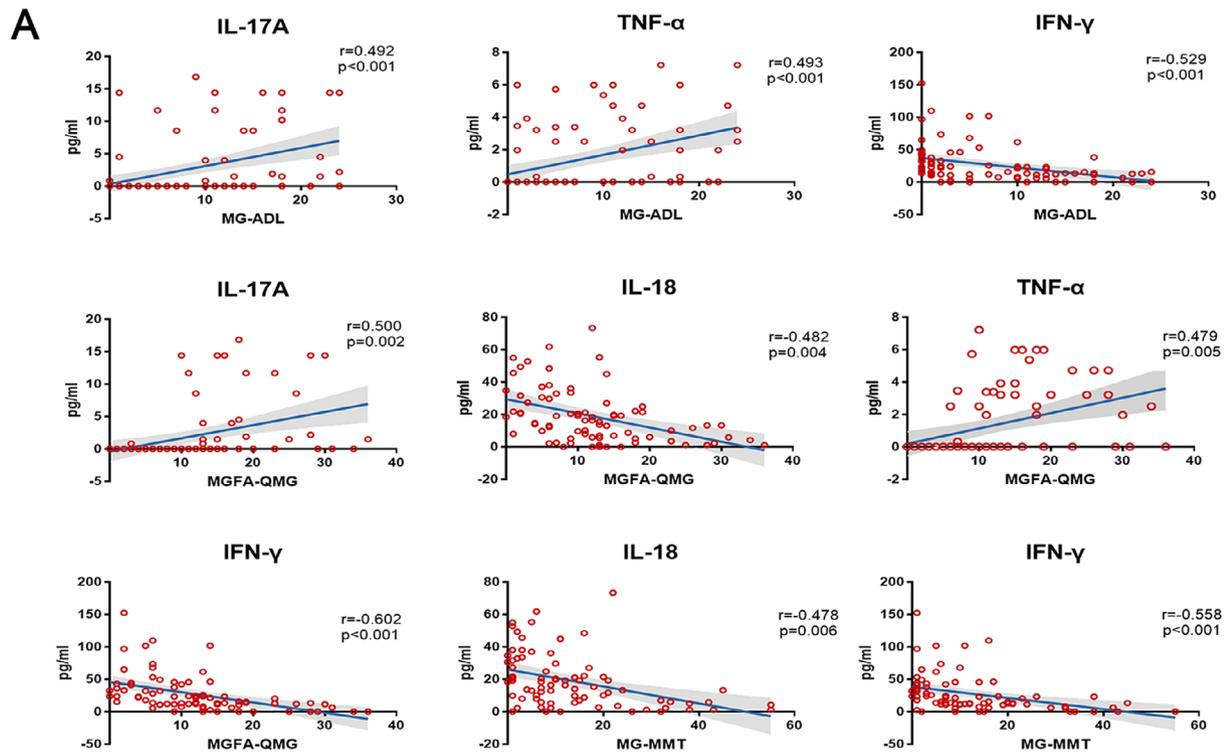
Figure 3. Nine significant correlations were revealed by analysis between the CD4$^+$ T lymphocytes and cytokines with MG clinical scores (A). After multiple testing correction, regression coefficient of each variable was depicted by black dots (no significance) and red dots (significance). Seven biomarkers including Tregs, IL-2/4/17A/IFN-γ/TNF-α/GM-CSF had significant correlations with MG-ADL scale (B). MG, myasthenia gravis; MG-ADL, MG-activities of daily living.

Table 4. Multivariate analysis among CD4$^+$ T cells, cytokines, and MG-ADL score after adjustment of gender and age.

Variable	Coefficient	Up	Down	P	P adjust
Tnaive	−0.032	0.013	−0.077	0.474	0.778
TCM	0.096	0.14	0.052	0.033	0.778
TEM	−0.035	0.008	−0.079	0.414	0.778
TEMRA	−0.178	−0.058	−0.298	0.141	0.778
Th1	−0.049	0.036	−0.134	0.566	0.778
Th2	0.032	0.094	−0.031	0.612	0.778
Th9	0.142	0.231	0.053	0.113	0.778
Th17	0.128	0.224	0.032	0.185	0.778
Th17/1	0.162	0.306	0.018	0.263	0.778
Tfh	0.164	0.27	0.058	0.125	0.778
ICOS + Tfh	0.295	0.643	−0.052	0.397	0.778
ICOS-Tfh	0.168	0.283	0.053	0.146	0.778
Tfh1	0.093	0.425	−0.238	0.778	0.778
Tfh2	0.302	0.629	−0.025	0.357	0.778
Tfh17	0.48	0.693	0.266	0.026	0.728
Tnaive in Tfh	0.633	1.147	0.118	0.222	0.778
TCM in Tfh	0.181	0.302	0.06	0.139	0.778
TEMRA in Tfh	15.077	23.971	6.184	0.093	0.778
TEM in Tfh	0.645	1.665	−0.375	0.529	0.778
Treg	0.825	1.052	0.599	0	0
IL-1β	0.135	0.358	−0.088	0.547	0.778
IL-2	0.201	0.25	0.153	0	0
IL-4	0.208	0.264	0.152	0	0
IL-5	0.54	0.83	0.25	0.066	0.778
IL-6	0.064	0.149	−0.021	0.454	0.778
IL-9	0.183	0.364	0.003	0.312	0.778
IL-10	−0.333	−0.057	−0.61	0.231	0.778
IL-12p70	−1.556	−0.594	−2.517	0.109	0.778
IL-13	1.238	1.705	0.771	0.01	0.3
IL-17A	0.702	0.856	0.548	0	0
IL-18	−0.09	−0.043	−0.136	0.059	0.778
IL-21	−0.707	−0.157	−1.257	0.202	0.778
IL-22	0.058	0.136	−0.021	0.463	0.778
IL-23	−0.727	−0.438	−1.017	0.014	0.406
IL-27	0.462	0.607	0.317	0.002	0.062
IFN-γ	−0.116	−0.087	−0.144	0	0
TNF-α	1.539	1.887	1.191	0	0
GM-CSF	0.115	0.148	0.083	0.001	0.032

Immune biomarkers with significant changes were marked in red. MG-ADL, MG-activities of daily living; TCM, T central memory; TEM, T effect memory; TEMRA, T effect memory-ra.

TNF-α ($r = 0.493$, $P < 0.001$), and IL-17A ($r = 0.492$, $P < 0.001$) with MG-ADL score, four significant correlations of IFN-γ ($r = −0.602$, $P < 0.001$), TNF-α ($r = 0.479$, $P = 0.005$), IL-18 ($r = −0.482$, $P = 0.004$), and IL-17A ($r = 0.500$, $P = 0.002$) with MGFA-QMG, and two significant correlations of IFN-γ ($r = −0.558$, $P < 0.001$) and IL-18 ($r = −0.478$, $P = 0.006$) with MG-MMT. Significant correlations ($P < 0.05$) revealed by univariate analysis are illustrated in Figure 3A.

After adjusting for sex and age and particularly focusing on the most commonly used questionnaire in MG trials, MG-ADL,[27] we identified seven significant correlations in the proportions of Tregs (95% CI: 0.599–1.052%, $P < 0.001$), or in the levels of cytokines IL-2 (95% CI: 0.153–0.25 pg/mL, $P < 0.001$), IL-4 (95% CI: 0.152–0.264 pg/mL, $P < 0.001$), IL-17A (95% CI: 0.548–0.856 pg/mL, $P < 0.001$), IFN-γ (95% CI: −0.144 to −0.087 pg/mL, $P < 0.001$), TNF-α (95% CI: 1.191–1.887 pg/mL, $P < 0.001$), and GM-CSF (95% CI: 0.083–0.148 pg/mL, $P = 0.032$) with MG-ADL. Regression Coefficients of each variable derived from the longitudinal data are depicted with black dots (no significance) and red dots (significance) (Fig. 3B, Table 4).

We then evaluated whether peripheral CD4$^+$ T, cytokine profile, and clinical scores dynamically changed in patients after the crisis. We plotted the individual variables of the MC cohort by multiple visits using PCA. The plots revealed a distinguished clustering pattern in crisis from that in the other five time points (1 week, 1 month, 2 months, 3 months, 6 months off-ventilation) (Fig. 4).

Discussion

Cross-sectional studies had proven the dominating role of proinflammatory CD4$^+$ T cells in MG, however, the in-depth phenotyping and immune dynamics remained unclear. Besides, the heterogeneity in peripheral T-cell-mediated autoimmunity exists in MG patients with distinct clinical severity and course, in particular at the most severe stage of MC, making it an attractive target for precision therapeutics. Currently, there are very few studies regarding the potential novel biomarkers for MG. In this study, we longitudinally investigated 20 CD4$^+$ T subpopulations and 18 cytokines using in-depth immune profiling in a cohort of patients experienced MC. With the high-throughput evaluation of serum biomarkers, we identified a distinguished pattern in crisis with highly elevated proinflammatory CD4$^+$ T subsets and cytokine cascade. This study also identified Tregs, IL-2, IL-4, IL-17A, IFN-γ, TNF-α, and GM-CSF as potential biomarker candidates for monitoring MG.

From peripheral CD4$^+$ T profile in MC, a re-balancing immune phenomenon was of note with a trend toward the inflammatory state. The numbers of all lymphocytes were significantly lower in MG with/without crisis compared with controls, supporting the effects of immunosuppressants therapy in high proportions of patients. In addition, patients in crisis had increased Tregs, which were considered to be critical in the maintenance of peripheral immune tolerance and can be promoted by intravenous immunoglobulin therapy during the crisis.[28] Apart from the elevated Tregs, a cluster of proinflammatory cytokines as well as increased frequencies of Tfh17 were demonstrated. A vigorous inflammatory response from a relatively small amount of T cells could represent the consequence of immune battling. Located in the
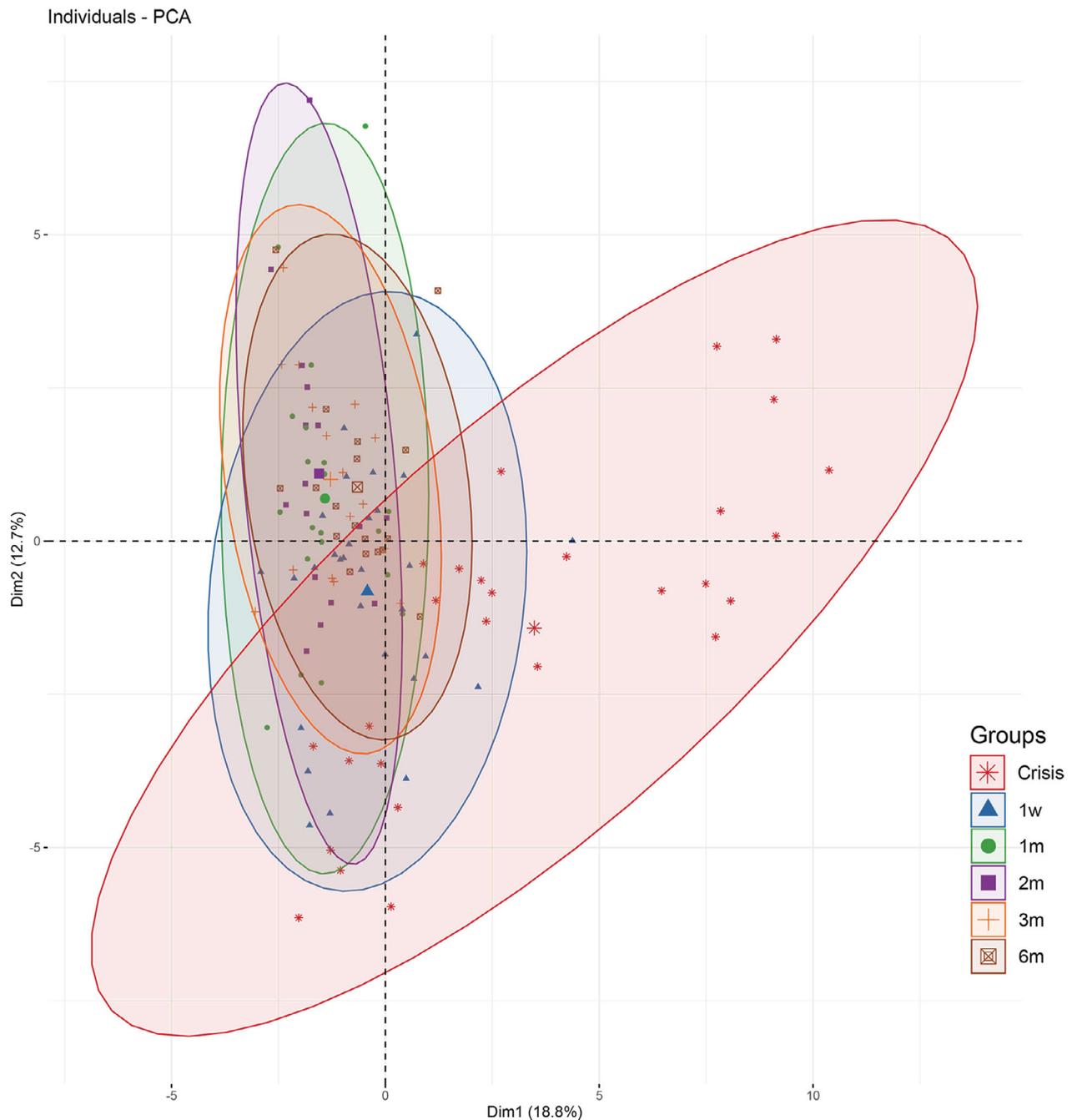
Figure 4. A PCA analysis of the correlations among peripheral CD4$^+$ T cells, cytokine profile, and clinical scores at different phases from crisis to 6 months off-ventilation. Clinical scores evaluated in this cohort included MG-ADL, MG-QoL15, MGFA-QMG, and MG-MMT. Positive correlated variables point to the same side of the plots, and vice versa. The orthogonal axes of Dim1 and Dim2 represent variation differences among all variables. A skewed distribution derived from the crisis cohort represented a distinct clustering pattern from that at other phases. PCA, principle component analysis; MG-ADL, MG-activities of daily living; QoL-15, MG Quality of Life 15; MGFA-QMG, MGFA-quantitative MG test; MG-MMT, MG manual muscle test.

lymphoid follicles, Tfh cells are a cluster of unique CD4$^+$ T-cell subsets that are essential for initiation and maintenance of the germinal follicle (GCs), thus playing important roles in affinity maturation of the antibodies and development of long-lived humoral immunity in MG.[29] Our findings highlighted an inquiry in Tfh17 cells and their functions in association with the status of crisis in a larger cohort. Additionally, longitudinal follow-ups of

cytokines revealed a significant elevation in crisis followed by a marked decrement within 1-week off-ventilation, indicating a "time-window" for rescue immune therapy in MC patients to reduce the in-hospital mortality and intensive care unit monitoring.

During T-cell differentiation, a network of cytokines and related molecules play a pivotal role. Although the exact mechanisms remain unknown, Th2-related cytokines such as IL-2, IL-4, IL-5, and IL-6 can stimulate plasma cells and B cells, thus facilitating antibody secretion.[30] Additionally, IL-6 facilitates the differentiation of CD4^{+} T cells into Th17 cells. IL-17A, IL-22, and GM-CSF are mainly derived from Th17 cells and involved in recruiting more inflammatory cells.[31] Previous studies of anti-IL-17 antibodies in treating psoriasis revealed a marked efficacy, however, it was not sufficient to change the clinical endpoint.[32] Relatively speaking, IL-6 is involved in multiple pathways of adaptive immunity with context-dependent pro and anti-inflammatory properties. As an IL-6 receptor inhibitor, Tocilizumab blocks the IL-6-mediated signal transduction. It is utilized to treat autoimmune disorders such as giant cell arteritis, systemic sclerosis, and neuromyelitis Optica Spectrum Disorder.[33–35] A therapeutic rationale for targeting multiple proinflammatory cell lineages and/or related cytokines requires future investigation for the patients in crisis.

Longitudinally speaking, in the prospective MC cohort, a coordinated upregulation of Th1, Th17, and TEM subsets, as well as a downregulation in most T follicular cells and Tregs were identified. This finding is in concordance with the principles of CD4^{+} T-cell differentiation.[36] Apart from the cytokine cascade in crisis, significant correlations in Th1-, Th2-, and Th17-related cytokines with clinical scores were identified in accordance with a re-balancing mechanism in peripheral T-cell lineages. Of note, Th1-related (IL-12p70, IL-18, and IFN-γ) and Th17-related cytokines (IL-21 and IL-23) were persistently elevated after 1-week off-ventilation in follow-ups, among which IL-18 level manifested negative correlations with clinical scores.

It is an unexpected finding that IFN-γ was elevated in 6 months off-ventilation. It is probably due to the anti-inflammatory properties of IFN-γ.[37,38] Evidence has been accumulated in recent years that supports the concept of a dual role IFN-γ in inflammation. These include induction of anti-inflammatory cytokine production, activation of apoptosis, and interference with the signal transduction machinery by induction of suppressors of cytokine signaling. To be noticed, Th2-related cytokines, IL-4 and IL-13, were significantly decreased in 6 months off-ventilation, which were previously characterized as the inducers in pathogenic antibody production.[39,40]

There are some limitations in our study. First, this study is mainly correlative. Although we revealed a dynamic alteration of distinct CD4^{+} T lymphocytes and cytokines at different stages in or after the MC, the causations between them are not clear. The impact of the combinations of thymoma, different immunosuppressants on the peripheral immune profile is not well-controlled. Second, due to the heterogeneity of MG participants including thymoma concurrent, different antibody profile, and immune therapy regimens, we have many confounders in analyzing the immune biomarkers in subgroups. Third, we introduced some selection bias by recruiting and stratifying patients, since patients referred to our diagnostic center were chosen from each referring hospital. The majority of patients had immunosuppressants treatment and harbored decreased CD4^{+} T-cell counts. Fourth, the duration of this longitudinal observation just extended to 6 months after coming off MV. Many CD4^{+} T subsets and cytokines may exhibit a late response but still strong correlations with the clinical severity. Therefore our results represent short-term peripheral immune dynamics in MC. In addition, a prospective cohort study of peripheral immune profile in generalized MG patients covered from the disease onset to the impeding or manifest crisis will give more information about the pre-crisis state. The reliability of the methodology of using fresh blood specimens for flow cytometry analysis in this study requires further testing in the multicenter analysis.

In conclusion, we have demonstrated active proinflammatory peripheral CD4^{+} T subsets and cytokine cascade in patients in crisis using in-depth immune profiling. Significant correlations between Treg subsets, Th1-, Th2-, and Th17-related cytokines and clinical scores were revealed. Thus, immune profiling-based biomarker development and precision therapeutic applications are to be further investigated in patients with MC.

Acknowledgments

Conflict of Interests

None declared.

Ethics Approval

The study was approved by the institutional review board of General Huashan hospital Fudan University (2016-271). Written informed consent was obtained from each

study participant before sample collection. All study procedures were performed in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki.

Provenance and Peer Review

Not commissioned; externally peer reviewed.

Data availability statement

Data are available upon reasonable request to the corresponding author.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Cross-sectional comparisons of CD4$^+$ T-cell subpopulations and cytokines between thymoma-associated MC versus non-MC GMG.

Table S2. Cross-sectional comparisons of CD4$^+$ T-cell subpopulations and cytokines between non-thymoma-associated MC versus non-MC GMG.

Table S3. Comparisons of immune biomarkers in thymoma-associated versus non-thymoma-associated MC, and thymoma-associated versus non-thymoma-associated non-MC GMG patients.

Table S4. Comparisons of immune biomarkers between MC patients and non-MC GMG patients with immunosuppressants.

Table S5. Paired comparisons between the immune biomarkers between healthy controls and that in MC cohort from crisis state, 1-week/1-month/2-months/3-months/6-months off-ventilation.