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Unbalanced expression of membrane-bound and soluble inducible costimulator and programmed cell death 1 in patients with myasthenia gravis

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ABSTRACT

This study aimed to investigate the possible functions and mechanisms of positive and negative costimulatory molecules in the pathological process of myasthenia gravis (MG). The expression levels of membrane-bound inducible costimulator (ICOS) and programmed cell death 1 (PD-1) in peripheral blood T cells, their corresponding ligands ICOSL and PDL-1 on B cells, and their soluble forms (sICOS, sPD-1, sICOSL, and sPDL-1) in plasma were detected in patients with untreated-stage MG (USMG) and remission-stage MG (RSMG). The results showed that the expression levels of membrane-bound ICOS and PD-1 in the peripheral blood T cells of the USMG group and their corresponding ligands ICOSL and PD-L1 on B cells were significantly increased compared to those in the RSMG group and healthy controls (HCs). The levels of sICOSL and sPD-1 were significantly upregulated in USMG patients compared to those in the RSMG and HC groups, while the levels of sICOS and sPD-L1 were not different. The expression of PD-L1 on $CD19^+$ B cells was positively correlated with the concentrations of AchR Ab in the USMG group. The expression of ICOS and PD-1 in $CD4^+$ T cells and the expression of ICOSL and PD-L1 on $CD19^+$ B cells were positively correlated with the quantitative myasthenia gravis (QMG) scores in the USMG group. Also, in the USMG group, the plasma levels of sICOSL and sPD-1 were positively correlated with the QMG scores. In addition, the percentage of peripheral blood follicular helper T (Tfh) cells in the USMG group was positively correlated with ICOS and PD-1 expression on $CD4^+$ T cells and ICOSL and PD-L1 expression on $CD19^+$ B cells. There were positive correlations between sICOSL and sPD-1 levels and the percentage of peripheral blood Tfh cells and plasma interleukin-21 (IL-21) levels in the USMG group. The results suggest that the positive ICOS/ICOSL and negative PD-1/PD-L1 costimulatory molecule pairs participate in the pathological process of MG. Abnormal sICOSL and sPD-1 expression might interfere with the normal signal transduction of ICOS and PD-1 on Tfh cells, causing excessive activation of Tfh cells and promotion of disease progression. sICOSL and sPD-1 have potential value in monitoring MG disease states.

1. Background

Myasthenia gravis (MG) is an acquired autoimmune disease, mediated by autoantibodies, that involves postsynaptic acetylcholine receptors in neuromuscular junctions or related functional molecules. Its major clinical manifestation is weakness and morbid fatigue of the involved skeletal muscles [1]. The etiology and pathogenesis of MG are still not completely clear. MG is considered a classic humoral immune disease. Self-reactive B cells and their secreted pathogenic antibodies, including acetylcholine receptor antibody (AChR Ab), play critical roles in the development of MG [2]. However, antibody/B-cell depletion therapy does not relieve symptoms in all MG patients, and patients without autoantibodies cannot be completely explained by humoral immunity. Increasing evidence has indicated that the production of

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pathogenic antibodies is inseparable from the function of $CD4^+$ T helper (Th) cells. Different T cell subsets and their secreted cytokines play important roles in different stages of the MG pathological process [3–7]. Costimulatory molecules closely associated with T-cell activation during immune responses have received much attention in recent years. How these positive and negative costimulatory molecules change during the pathological process of MG, their possible mechanisms, and their roles in disease outcome should be further investigated.

Inducible costimulator (ICOS)/inducible costimulator ligand (ICOSL) are a pair of positive costimulatory molecules belonging to the B7/CD28 costimulator family. ICOS is expressed mainly on the surface of activated or memory T cells. Its ligand, ICOSL, is mainly expressed in the primary and secondary follicles of lymph nodes [8]. ICOS/ICOSL mainly participates in regulating different stages after T-cell activation. Their main functions include promotion of the formation of the germinal center (GC) and conversion between antibody types [9]. Programmed death 1 (PD-1)/programmed death 1 ligand (PD-L1) is an important pair of negative costimulatory molecules belonging to the B7 family. PD-1 is mainly expressed on $CD4^+$ and $CD8^+$ T cells, natural killer T cells, and monocytes [10]. PD-L1 is mainly expressed on B cells, dendritic cells, activated T cells, and non-hematopoietic cells. The PD-1/PD-L1 interaction has important regulatory functions at the initial stage of self-reactive T-cell activation and amplification and in the secondary immune response; it can also inhibit B-cell amplification and differentiation and antibody-type conversion [10]. ICOS and PD-1 expression on activated T lymphocytes play positive and negative regulatory roles in immune responses, respectively, and have important functions in maintaining the self-stable state of the immune system to ensure the host's adaptive immunity. ICOS/ICOSL and PD1/PD-L1 have membrane-bound and soluble forms. The soluble molecules bind to their corresponding membrane-bound molecules through direct receptor/ligand interactions to carry out their regulatory roles [11]. Abnormal expression of these membrane-bound and soluble molecules occurs at the same time in different autoimmune diseases [12–14]. At present, the specific functions and mechanisms of the ICOS/ICOSL and PD-1/PD-L1 pathways in MG immune disorders and injuries are unclear. It is necessary to evaluate whether the abnormal expression or abnormal changes of these molecules (including their membrane-bound and/or soluble forms) are related to the onset and severity of MG and the pathways involved in their functions.

Follicular helper T (Tfh) cells are a recently discovered $CD4^+$ T-cell subset. They are localized to the GC of lymphoid follicles and participate in humoral immune responses [15]. Tfh cells express various types of surface molecules including Bcl-6, CXCR5, ICOS, PD-1, CD40L, and SAP, and they can secrete IL-21 and IL-4 [16]. The ICOS and PD-1 pathways play critical roles in the steady state and function of Tfh cells. High levels of ICOS on Tfh cells bind to ICOSL on the surface of B cells to initiate downstream signals that promote Tfh cell differentiation, development, and transport; induce Tfh cells to secrete IL-21; and assist in B-cell proliferation, differentiation, immunoglobulin production and conversion to corresponding types [15]. By interacting with its ligand, PD-1 plays an important role in the establishment and maintenance of self-immune tolerance by delivering inhibitory signals [17]. However, PD-1 deficiency can result in the reduced production of Tfh-cell-related cytokines and the death of GC cells, which further influence the number and quality of long-lived plasma cells [18]. Abnormal numbers of, and dysfunctional, Tfh cells are closely associated with many autoimmune diseases, including systemic lupus erythematosus (SLE), Sjögren's syndrome, rheumatoid arthritis (RA), and MG [15]. In thymoma patients, high Tfh-cell expression is positively correlated with the severity of MG [19]. The expression levels of ICOS and PD-1 were upregulated in the circulating Tfh-Th17 cells of MG patients. However, it is not clear why upregulation of both positive and negative costimulatory molecules [20] still leads to disease development, and the mechanisms underlying this occurrence require elucidation.

This study detected ICOS and PD-1 on the surface of peripheral-blood T cells from MG patients, ICOSL and PD-L1 on the surface of their $CD19^+$ B cells, ICOS/PD-1 coexpression on the surface of their Tfh cells, and the expression levels of related soluble molecules (sICOS, sICOSL, sPD-1, and sPD-L1) in their plasma to investigate the functions, mechanisms, and clinical significance of the ICOS/ICOSL and PD-1/PD-L1 pathways in the development of MG.

2. Materials and methods

2.1. Study subjects

This study was reviewed and approved by the Ethics Committee at The First Affiliated Hospital of Soochow University, China. All subjects signed informed consent. Between May 2016 and December 2017, 72 patients with MG in the untreated stage (USMG) at The First Affiliated Hospital of Soochow University were selected. Confirmation of USMG was based on typical clinical manifestations as well as the fatigue test, neostigmine test, repetitive nerve electrical stimulation, and AChR-Ab results. Patients who also had other autoimmune diseases and/or had received glucocorticoids, immunosuppressants, intravenous immunoglobulins, or plasma-exchange therapy in the past 3 months were excluded. Assessment of disease severity was determined by the quantitative MG (QMG) score, and all patients received standard treatment after admission into the hospital. Six months after disease onset and treatment, 23 of these patients achieved myasthenia gravis remission (RSMG) and participated in the follow-up. These patients all conformed to the definition of remission in the 2016 International consensus guidance for the management of myasthenia gravis [21]. Concurrently, 25 age- and gender-matched volunteers from the hospital's physical examination center were enrolled in the healthy control (HC) group. The patients' demographic and clinical characteristics are shown in Table 1.

2.2. Sample processing

With informed consent, 4 ml of fasting venous blood were collected in EDTA-anticoagulant tubes from the MG patients and healthy volunteers in the morning; 1 ml was used for the flow cytometric analysis of lymphocytes. The remaining 3 ml were transferred into a dry test tube and centrifuged at 4 °C and 230 *g* for 20 min, after which the top-layer plasma sample was aliquoted and frozen at −80 °C for future use.

2.3. Immunofluorescent labeling and flow cytometric analysis

Peripheral blood (50 μl) was collected from each subject, and the following fluorescently labeled antibodies were added, followed by incubation at room temperature for 30 min: CD4-PC7, ICOS-FITC, CXCR5-PE, PD-1-PC5, CD19-FITC, and isotype-matched control IgG antibodies (all purchased from BioLegend, San Diego, CA, USA). For red-blood-cell lysis and cell fixation, 200 μl of red-blood-cell lysis buffer (Beckman Coulter, Brea, CA, USA) was added, and the mixtures were incubated at 37 °C for 10 min. Finally, each sample was washed with 1 ml of PBS, resuspended in 500 μl of PBS, and analyzed using a flow cytometer (Beckman Coulter, Brea, CA, USA). FlowJo 10.0 software (FlowJo, LLC, Ashland, OR, USA) was used to analyze the raw flow cytometry data. At least 50,000 cells were analyzed in each sample.

Table 1
Baseline clinical characteristics of MG patients and healthy controls.

	USMG	RSMG	HC	*P* value
N	72	23	25	
Age, Median [Range]	52 [20–79]	52 [26–70]	48 [30–83]	0.9070
Female, n (%)	39 (54.2%)	12 (52.2%)	14 (56.0%)	0.9174
Type				
GMG	46	15	Na	0.9079
OMG	26	8	Na	
Onset age, n (%)				
< 40	18 (25.0%)	5 (21.7%)	Na	0.7506
≥40	54 (75.0%)	18 (78.3%)	Na	
Presence of thymoma, n (%)	17 (23.6%)	7 (30.4%)	Na	0.5120
Anti-AChR-Ab-positive, n%	53 (73.6%)	18 (78.3%)	Na	0.6550
QMG score, Median [Range]	12 [2–28]	16 [2–28]	Na	0.4655
Therapy, n (%)				
Pyridostigmine	66 (91.7%)	22 (95.7%)	Na	> 0.9999
Glucocorticoid	59 (81.9%)	21 (91.3%)	Na	0.3476
Immunosuppression				
Azathioprine	27 (37.5%)	10 (43.48%)	Na	0.6306
Cyclophosphamide	12 (16.7%)	7 (30.4%)	Na	0.2288
IV immunoglobulin	35 (48.6%)	15 (65.2%)	Na	0.2308
Thymectomy	23 (31.9%)	12 (52.2%)	Na	0.0891

Note: USMG, untreated stage myasthenia gravis; RSMG, remission stage myasthenia gravis; HC, healthy controls; OMG, ocular myasthenia gravis; GMG, generalized myasthenia gravis; QMG score, quantitative myasthenia gravis score.

2.4. ELISAs

The concentrations of anti-AChR Ab in USMG patients were determined by ELISA using the Human Acetylcholine Receptor Autoantibody ELISA Kit (Tianjin RSR Biotechnology Co., Ltd., Tianjin, China,). Blood samples from 23 MG patients who participated in the follow-up were collected when they were in the USMG and RSMG phases. Plasma levels of sICOS, sICOSL, sPD-1, sPD-L1, and IL-21 were analyzed using ELISAs (the human sICOS, sICOSL, sPD-1, sPD-L1, and IL-21 reagent kits were obtained from Shanghai Kang Lang Biological Technology Co., Ltd., Shanghai, China). The ELISAs were performed according to the manufacturer's instructions.

2.5. Statistical analyses

Statistical analyses were performed using R 3.5.1 (https://cran.r-project.org) and GraphPad Prism 7 software (GraphPad, Inc., San Diego, CA, USA). Quantitative data are expressed as medians (interquartile ranges). Comparisons between 2 groups of independent samples were analyzed using the Mann–Whitney *U* test. The Kruskal–Wallis H test was used to compare samples among multiple groups, while the Bonferroni-corrected Mann–Whitney *U* test was used to compare groups. Qualitative data are expressed as numbers and percentages. The Chi-square test or Fisher's exact test was used to compare the relationships between qualitative variables. The nonparametric Spearman correlation was used to examine the relationship between two continuous variables. A p-value < .05 was considered statistically significant.

3. Results

3.1. ICOS/ICOSL and PD-1/PD-L1 expression was increased on the peripheral blood lymphocytes of MG patients

ICOS/ICOSL and PD-1/PD-L1 expression on the peripheral blood lymphocytes of patients in the USMG, RSMG, and HC groups was analyzed by flow cytometry. The expression of ICOS and PD-1 on $CD4^+$ T cells in the USMG and RSMG groups was significantly higher than that in the HC group. Compared with the RSMG group, the expression of ICOS and PD-1 on the $CD4^+$ T cells in the USMG group was significantly increased (Fig. 1A, E). ICOSL and PD-L1 expression on $CD19^+$ B cells was significantly elevated in the USMG and RSMG groups compared with the HC group. The expression of ICOSL and PD-L1 on $CD19^+$ B cells in the USMG group was higher than that in the RSMG group (Fig. 1C, E).

Correlation analysis showed that the expression of ICOS on $CD4^+$ T cells was positively correlated with the expression of PD-1 in the USMG group, and the expression of ICOSL on $CD19^+$ B cells was positively correlated with PD-L1 expression (Fig. 1B, D). However, no such correlation was observed in the RSMG group.

3.2. sICOSL and sPD-1 levels were elevated in USMG patients

To investigate the potential roles of soluble costimulatory molecules, we collected blood samples from 23 USMG and RSMG patients and performed ELISA analysis. The levels of plasma sICOSL and sPD-1 in the USMG group were significantly higher than those in the RSMG and HC groups. There was no significant difference between the RSMG group and the HC group (Fig. 2B, C). sICOS and sPD-L1 levels were not significantly different between the USMG, RSMG, and HC groups. (Fig. 2A, D).

Spearman correlation analysis showed that the levels of sICOS and sPD-1 in the 3 groups were not correlated with ICOS and PD-1 expression on $CD4^+$ T cells. Similarly, in all 3 groups, the sICOSL and sPD-L1 levels were not correlated with the expression of ICOSL and PD-L1 on $CD19^+$ B cells.

3.3. Clinical correlation data

To explore the relationship between ICOS/ICOSL and PD-1/PD-L1 expression in peripheral blood lymphocytes and clinical indices in MG patients, comparative evaluation of subgroups and correlation analysis

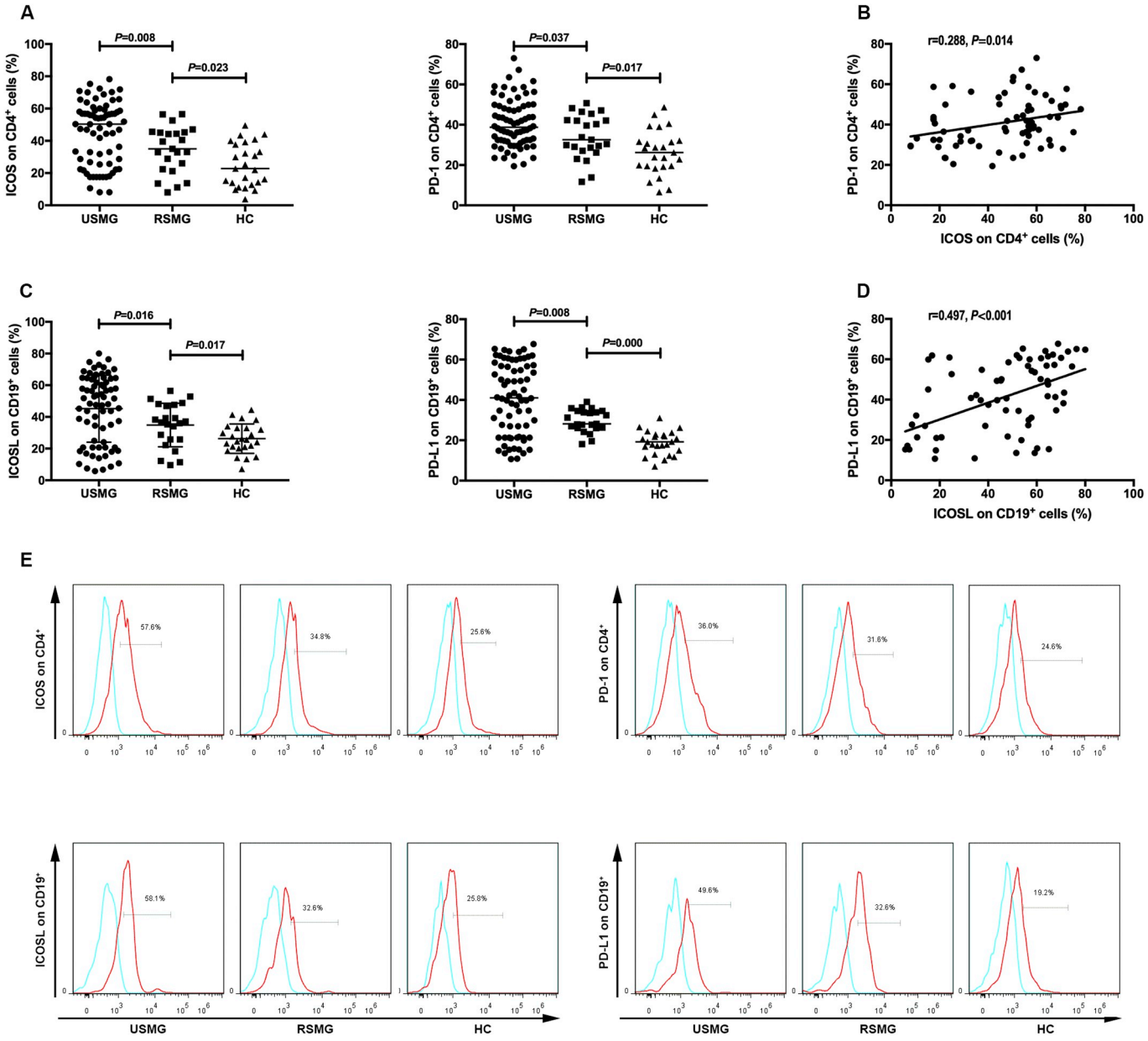

Fig. 1. ICOS/ICOSL and PD-1/PD-L1 expression on the peripheral blood lymphocytes of MG patients.
A. Comparison of ICOS and PD-1 expression on $CD4^+$ T cells in the USMG, RSMG and HC groups.
B. Linear correlation between ICOS and PD-1 expression on $CD4^+$ T cells in the USMG group.
C. Comparison of ICOSL and PD-L1 expression on $CD19^+$ B cells in the USMG, RSMG and HC groups.
D. Linear correlation between ICOSL and PD-L1 expression on $CD19^+$ B cells in the USMG group.
E. Representative expression of ICOS and PD-1 on $CD4^+$ T cells and ICOSL and PD-L1 on $CD19^+$ B cells in the USMG, RSMG, and HC groups. The red lines indicate specific staining results measured by flow cytometry, and the blue lines indicate isotype controls.

of laboratory parameters and clinical manifestations were performed.

The expression of ICOS and PD-1 on $CD4^+$ T cells and ICOSL and PD-L1 on $CD19^+$ B cells was significantly higher in the USMG group patients with GMG than in the group with OMG and significantly higher in those with thymoma than without. The levels of sICOSL and sPD-1 were significantly increased in USMG patients with GMG compared to patients with OMG, and those of the patients with thymoma were significantly increased compared to those without thymoma.

The expression of PD-1 on $CD4^+$ T cells in late-onset patients was also significantly higher than that of early-onset patients in the USMG

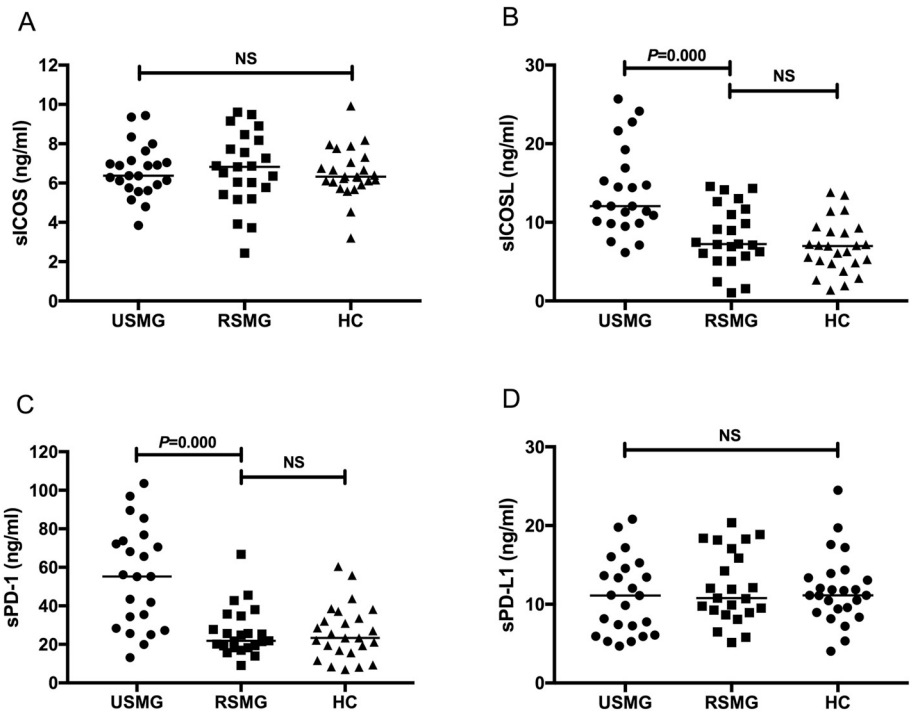

Fig. 2. Comparison of sICOS, sICOSL, sPD-1 and sPD-L1 plasma levels from 23 MG patients and 25 HCs. A. sICOS levels in the USMG, RSMG and HC groups. B. sICOSL levels in the USMG, RSMG and HC groups. C. sPD-1 levels in the USMG, RSMG and HC groups. D. sPD-L1 levels in the USMG, RSMG and HC groups. NS: no significance.

group. However, there were no other significant differences between subgroups based on gender, age of onset, and AchR-Ab status (Table 2). In addition, in the RSMG group, there were no statistically significant differences between the above-mentioned indicators among the subgroups.

Correlations between laboratory parameters and clinical data were analyzed. In the USMG group, the expression of PD-L1 on $CD19^+$ B cells was positively correlated with the concentration of AchR Ab, and there was a positive correlation trend between levels of sICOSL and sPD-1 and the concentration of AchR Ab. The expression of ICOS and PD-1 on $CD4^+$ T cells and the expression of ICOSL and PD-L1 on $CD19^+$ B cells were positively correlated with the QMG scores. Furthermore, sICOSL and sPD-1 levels in the USMG group were also positively correlated with the QMG scores (Table 3 and Fig. 3). Correlation analysis of the RSMG group did not show similar results.

3.4. Correlation with Tfh

Tfh cells are the most important subset of $CD4^+$ T cells that help B cells produce antibodies, and recent studies have confirmed that Tfh cells are associated with MG pathogenesis. Therefore, we evaluated the percentages of $CD4^+$ $CXCR5^+$ $ICOS^+$ $PD\text{-}1^+$ T cells and the levels of IL-21 in the peripheral blood of MG patients and performed correlation analyses.

The percentages of peripheral blood Tfh cells in the USMG and RSMG groups were significantly higher than that in the HC group, and the Tfh cells of the USMG group were significantly elevated compared with those RSMG group. Although plasma IL-21 levels were significantly higher in the USMG group than in the RSMG and HC groups, there was no significant difference between the RSMG and HC groups (Fig. 4). Subgroup analyses found that the percentage of peripheral blood Tfh cells and plasma IL-21 levels were significantly higher in USMG group patients with GMG than those with OMG, and those of the patients with thymoma were also significantly higher than those without thymoma. However, no significant differences were observed between subgroups based on gender, age of onset, and AchR-Ab status (Table 4).

Spearman correlation analyses indicated that the percentage of peripheral blood Tfh cells in the USMG group was positively correlated with the expression of ICOS and PD-1 on $CD4^+$ T cells and the expression of ICOSL and PD-L1 on $CD19^+$ B cells. Furthermore, the percentage of peripheral blood Tfh cells in the USMG group was positively correlated with plasma IL-21 levels, AchR-Ab concentrations, and QMG scores. Plasma IL-21 levels in the USMG group were positively correlated with the expression of ICOSL and PD-L1 on $CD19^+$ B cells and QMG scores (Fig. 5). Interestingly, we also found positive correlations between sICOSL and sPD-1 levels and the percentage of peripheral blood Tfh cells and plasma IL-21 levels in the USMG group (Table 3 and Fig. 6). However, we did not find similar significant correlations in the RSMG group.

4. Discussion

In this study, we found that expression of the respective positive and negative costimulatory molecules ICOS and PD-1 on the $CD4^+$ T cells of patients in the USMG group was significantly increased compared with that in the HC group. Similarly, ICOSL and PD-L1 expression on $CD19^+$ B cells was significantly higher than that in the HC group. Although the expression of these molecules significantly decreased after treatment, their levels were still higher than those in the HC group. In addition, in the USMG group, the expression of these molecules is related to the severity of MG. These results suggest that the ICOS/ICOSL and PD-1/

Table 2
ICOS/ICOSL and PD-1/PD-L1 expression on leukocyte subsets and plasma levels of soluble molecules in the USMG subgroups.

Group	ICOS on $CD4^+$ cells (%)	ICOSL on $CD19^+$ cells (%)	PD-1 on $CD4^+$ cells (%)	PD-L1 on $CD19^+$ cells (%)	sICOS (ng/ml)	sICOSL (ng/ml)	sPD-1 (ng/ml)	sPD-L1 (ng/ml)
Anti-AChR Ab								
Positive	50.33 [24.63–60.23]	48.19 [20.70–64.49]	41.40 [32.26–50.52]	41.98 [21.59–57.53]	6.63 [5.84–7.26]	12.15 [10.07–19.83]	62.15 [27.70–79.04]	9.02 [6.05–13.48]
Negative	52.29 [28.69–57.12]	52.05 [30.69–40.94]	36.50 [30.69–40.94]	34.56 [19.95–52.57]	6.11 [5.66–7.44]	10.88 [9.67–14.84]	55.12 [35.37–60.44]	14.55 [9.65–17.91]
Gender								
Male	51.90 [24.35–64.54]	43.70 [22.35–54.01]	41.00 [32.40–50.95]	40.60 [24.32–59.56]	6.89 [5.14–7.63]	14.48 [11.30–16.91]	43.44 [25.01–76.89]	11.11 [5.90–13.43]
Female	47.90 [25.72–58.60]	57.11 [20.70–64.69]	37.40 [31.60–47.60]	41.29 [21.33–56.67]	6.37 [6.12–7.01]	11.15 [9.57–18.03]	60.44 [37.06–73.34]	10.52 [7.39–16.89]
Clinical type								
GMG	55.13 [35.48–61.13][a]	57.14 [37.25–64.69][a]	42.89 [32.26–52.21][b]	49.26 [27.53–60.55][b]	6.89 [5.91–7.63]	14.48 [11.41–21.64][a]	70.50 [43.44–85.47][a]	8.17 [6.10–13.37]
OMG	31.05 [17.50–55.95]	36.93 [20.70–53.48]	36.40 [31.10–42.51]	33.26 [21.33–45.83]	6.20 [5.25–6.95]	9.87 [7.71–11.75]	27.84 [21.36–50.01]	13.53 [7.24–15.84]
Onset age								
< 40	28.69 [21.65–62.68]	46.25 [29.78–62.47]	32.40 [29.15–42.30][c]	38.54 [27.24–51.15]	6.91 [5.87–9.50]	19.23 [11.18–24.22]	68.18 [38.66–87.84]	9.87 [7.51–14.15]
≥ 40	54.07 [32.98–58.65]	52.23 [20.70–64.21]	41.49 [35.39–50.52]	42.57 [21.33–59.90]	6.37 [5.70–77.07]	11.73 [9.76–14.86]	55.18 [26.88–74.54]	11.60 [5.92–14.73]
Thymoma								
With	55.95 [47.37–65.90][d]	64.10 [43.70–69.46][e]	49.30 [42.80–55.84][e]	54.79 [39.67–62.24][e]	7.04 [6.89–8.34]	21.64 [11.30–24.14][d]	72.11 [56.11–96.93][d]	8.17 [7.41–11.11]
Without	46.90 [22.40–57.79]	47.30 [20.70–57.90]	36.70 [31.60–43.70]	34.67 [21.33–54.33]	6.21 [5.61–6.95]	11.73 [9.57–14.46]	39.45 [26.05–69.29]	12.70 [5.97–15.09]

[a] $P < 0.01$ vs. OMG.
[b] $P < 0.05$ vs. OMG.
[c] $P < 0.01$ vs. ≥40.
[d] $P < 0.05$ vs. Without.
[e] $P < 0.01$ vs. Without.

PD-L1 pathways participate in the immunopathological process of MG.

One study showed that disease progression was significantly suppressed in ICOS gene knockout mice with experimental autoimmune MG (EAMG). The level of serum AChR-specific immunoglobulin was decreased, and the GC reaction was weakened in secondary lymphoid tissues [22]. These findings indicate that the ICOS/ICOSL pathway contributes to the development of MG via the induction of T-cell-mediated humoral immunity. To the best of our knowledge, the current study is the first to discover increased expression of ICOS/ICOSL in MG patient peripheral blood lymphocytes. Thus, we hypothesize that ICOSL overexpressed on $CD19^+$ B cells interacts with ICOS on $CD4^+$ T cells and enhances the ICOS/ICOSL signal to promote additional activation and proliferation of ICOS expression T cells, mediating their long-term survival; this process induces immune disorders and immune injury. At the same time, abnormal increases of ICOSL on B cells can cause excessive B-cell activation to secrete autoantibodies that can induce pathological injury.

The PD-1/PD-L1 pathway mediates negative costimulatory signals, playing an important role in the suppression of self-reactive T cells and maintenance of immune tolerance. Defects in the PD-1/PD-L1 signaling pathway can result in the development of autoimmune diseases [10]. In this study, the expression of PD-1 on $CD4^+$ cells and PD-L1 on $CD19^+$ B cells of USMG patients was significantly higher than that in the HC group, consistent with the results of Sakthivel et al. [23]. The expression of PD1/PD-L1 was upregulated in MG patients; however, their upregulated expression could not block and relieve the disease. Based on previous literature reports, we made the following speculations. (1) The PD-1/PD-L1 pathway has the dual functions of causing cell cycle arrest and promoting cell differentiation and proliferation. These functions are achieved through the activation of the Ras/Raf/MEK/Erk pathway. [24]. (2) The positive costimulatory pathway that promotes T-cell differentiation and maturation could be stronger than the negative signal. (3) The PD-1 signal transduction pathway is inhibited and/or disrupted to promote the development and progression of MG. However, it is unclear which mechanism plays a major role. Our group detected PD-1/ICOS coexpression on Tfh cells and investigated the possible functions of these soluble forms in the 2 positive and negative signal groups.

Tfh cells are considered the most powerful regulators of humoral immunity because of their core functions in facilitating B-cell activation and differentiation and antibody production. Studies have confirmed that the number and dysfunction of peripheral blood Tfh cells are associated with many humoral immunity-mediated diseases, including MG [20,25–29]. IL-21 is the major cytokine through which Tfh cells exert their effects and execute their functions. After blocking the function of IL-21 with an IL-21-neutralizing antibody, the AChR-Ab concentration in a Tfh- and B-cell coculture significantly decreased [30]. In the present study, the percentage of peripheral blood Tfh cells and plasma IL-21 levels were significantly increased in the USMG group, and the percentages of Tfh cells were positively correlated with plasma IL-21 level and AchR-Ab concentration. This is basically consistent with previous research results. However, our study showed that the percentages of peripheral blood Tfh cells in USMG patients were positively correlated with the expression of ICOS and PD-1 on $CD4^+$ T cells and the expression of ICOSL and PD-L1 on $CD19^+$ B cells. Plasma IL-21 levels were positively correlated with the expression of ICOSL and PD-L1 on peripheral blood $CD19^+$ B cells. Based on our results, we hypothesized that overexpression of the ICOS/ICOSL and PD-1/PD-L1 pathways may play a role in MG pathogenesis by affecting Tfh-cell and IL-21 functions.

Tfh cells express the ICOS positive costimulatory molecule and the PD-1 negative costimulatory molecule together. However, there have been no reports regarding the effect of increased expression of the

Table 3
Relationships between laboratory parameters in USMG patients.

		ICOS on $CD4^+$ cells (%)	ICOSL on $CD19^+$ cells (%)	PD-1 on $CD4^+$ cells (%)	PD-L1 on $CD19^+$ cells (%)	$ICOS^+PD1^+$ on $CD4^+CXCR5^+$ cells (%)	sICOSL (ng/ml)	sPD-1 (ng/ml)	sPD-L1 (ng/ml)	IL-21 (ng/ml)
ICOS on $CD4^+$ cells (%)	r		***0.2898***	***0.2882***	***0.2503***					
	P		***0.0135***	***0.0141***	***0.0339***					
	n		***72***	***72***	***72***					
ICOSL on $CD19^+$ cells (%)	r			0.1340	***0.4967***					
	P			0.2620	***< 0.0001***					
	n			72	***72***					
PD-1 on $CD4^+$ cells (%)	r				0.1661					
	P				0.1632					
	n				72					
sICOS (ng/ml)	r	−0.0059	0.3804	0.3202	0.2352	0.2411	0.2451	0.3261	−0.1374	0.3972
	P	0.9786	0.0733	0.1364	0.2800	0.2677	0.2597	0.1289	0.5320	0.0605
	n	23	23	23	23	23	23	23	23	23
sICOSL (ng/ml)	r	0.0613	0.3893	0.1364	0.2885	***0.5672***		0.3073	−0.1136	***0.498***
	P	0.7812	0.0663	0.5350	0.1818	***0.0048***		0.1537	0.6057	***0.0156***
	n	23	23	23	23	***23***		23	23	***23***
sPD-1 (ng/ml)	r	0.0662	***0.5000***	0.3221	0.2549	***0.5128***			−0.1334	***0.4832***
	P	0.7641	***0.0151***	0.1339	0.2404	***0.0123***			0.5440	***0.0195***
	n	23	***23***	23	23	***23***			23	***23***
sPD-L1 (ng/ml)	r	−0.0356	0.0148	−0.1206	−0.2055	0.1196				0.1502
	P	0.8720	0.9465	0.5837	0.3468	0.5869				0.4939
	n	23	23	23	23	23				23
$ICOS^+PD1^+$ on $CD4^+CXCR5^+$ cells (%)	r	***0.4223***	***0.3541***	***0.3065***	***0.4462***					***0.5860***
	P	***0.0002***	***0.0023***	***0.0088***	***< 0.0001***					***0.0033***
	n	***72***	***72***	***72***	***72***					***23***
IL-21 (ng/ml)	r	−0.2164	***0.5543***	0.3132	***0.6235***					
	P	0.3213	***0.0061***	0.1456	***0.0015***					
	n	23	***23***	23	***23***					

Spearman correlations were calculated using GraphPad Prism 7 (GraphPad Inc., San Diego, CA, USA). R-values are given in the first row, the corresponding *P* values are given in the second row, and the numbers of persons included are given for each analysis. Significant correlations are shown in bold, italic font.

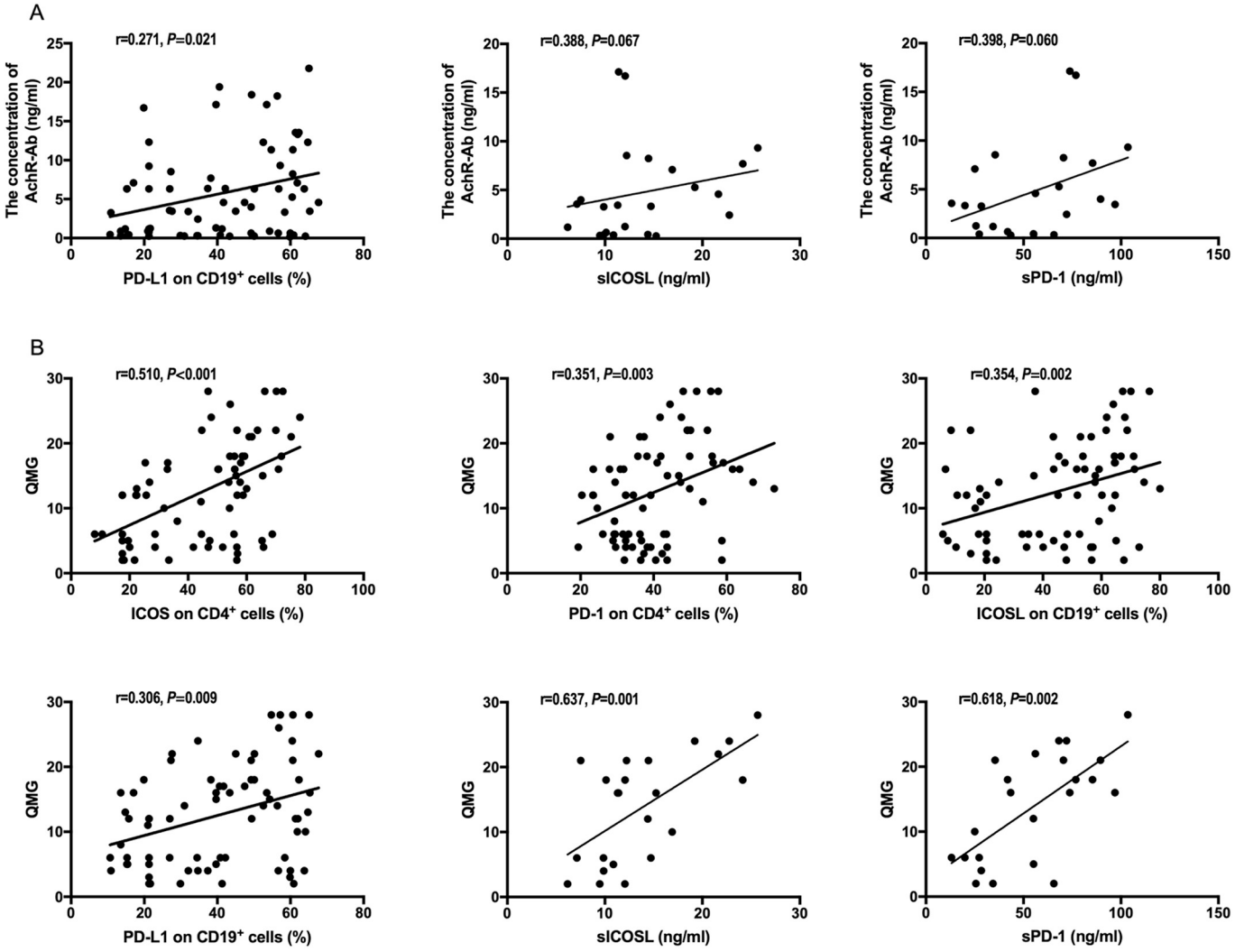

Fig. 3. Clinical correlation diagram.

A. Linear correlations between the concentration of AchR-Ab and the expression of PD-L1 on $CD19^+$ B cells (n = 72) and the plasma levels of sICOSL and sPD-1 in the USMG group (n = 23).

B. Linear correlations between QMG scores and the expression of ICOS/PD-1 on $CD4^+$ T cells, ICOSL/PD-L1 on $CD19^+$ B cells (n = 72) and the plasma levels of sICOSL and sPD-1 in the USMG group (n = 23).

ICOS/ICOSL and PD-1/PD-L1 pathways on Tfh-cell function. The receptors and ligands of costimulatory molecules exist not only in membrane-bound forms but also as soluble forms in the blood of MG patients and healthy people. These soluble molecules can bind to their corresponding membrane receptors/ligands to exert different biological effects. Studies have confirmed that sPD-1 and sPD-L1 inhibit the PD-1/PD-L1 pathway, enhance the effect of T cells, and are associated with autoimmune hepatitis and childhood autoimmune arthritis [31–33].

Therefore, to investigate the roles of these soluble molecules, we examined plasma sICOS, sICOSL, sPD-1, and sPD-L1 levels in MG patients. The results showed that in the USMG group, sICOSL and sPD-1 levels were significantly higher than in the RSMG and HC groups, and they were positively correlated with the percentage of peripheral blood Tfh cells and plasma IL-21 levels in the USMG group. Based on these findings, we hypothesized that sICOSL and sPD-1 participate in the immunopathological process of MG development by regulating or interrupting the function of Tfh cells. Hamel et al. [34] showed that ICOSL expressed on B cells was necessary for the formation and maintenance of GC and the production of Tfh cells and antibodies. This finding suggested that binding between the increased sICOSL in the peripheral blood and ICOS on Tfh cells could allow Tfh cells to receive persistent and continuous positive stimulation signals, causing their excessive activation and proliferation. Besides, sPD-1 could bind to PD-L1 on the membranes of T cells, B cells, or DCs to interfere with negative signals transduced by the PD-1/PD-L1 pathway, causing relative immune-system hyperactivity [35]. In this study, our results showed that the concentration of sPD-L1 was only 1/5 that of sPD-1. We speculated that the excess sPD-1 could interfere with the binding

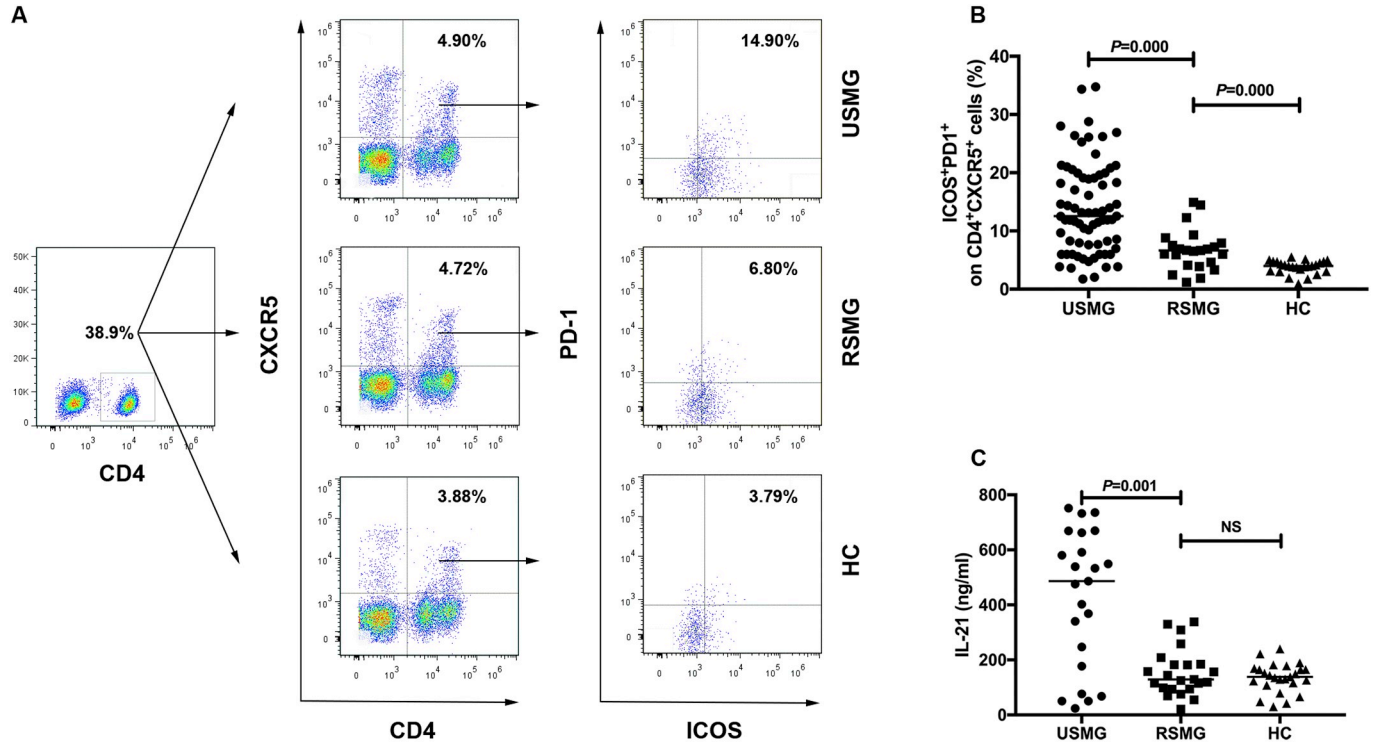

Fig. 4. Percentages of $CD4^+CXCD5^+ICOS^+PD1^+$ Tfh cells and IL-21 plasma levels in the peripheral blood of MG patients.
A. Gating strategy of Tfh cells. Lymphocytes in peripheral blood mononuclear cells (PBMCs) were gated according to forward and side scatter, and then $CD4^+$ T cells were identified. The $CXCR5^+$ T cells were circled by $CD4^+$ T cells. Subsequently, Tfh cells were gated on ICOS and PD-1 in $CD4^+CXCR5^+$ T cells, and the percentages of Tfh cells were calculated as the proportion of $ICOS^+PD\text{-}1^+$ cells on $CD4^+CXCR5^+$ T cells.
B. Comparison of the percentages of $CD4^+CXCD5^+ICOS^+PD1^+$ Tfh cells in the USMG, RSMG and HC groups (n = 72).
C. Comparison of plasma IL-21 levels in the USMG, RSMG and HC groups (n = 23).

Table 4
Tfh percentages and plasma IL-21 levels in the USMG subgroups.

Group	$ICOS^+PD1^+$ on $CD4^+CXCR5^+$ cells (%)	IL-21 (ng/ml)
Anti-AChR Ab		
Positive	13.30 [7.45–20.61]	536.14 [317.07–669.17]
Negative	11.66 [7.25–13.00]	177.07 [37.12–491.53]
Gender		
Male	13.90 [7.78–20.26]	475.49 [247.30–732.26]
Female	11.87 [6.99–19.09]	512.71 [101.48–588.34]
Clinical type		
GMG	13.53 [9.40–21.26][b]	533.13 [368.1–669.82][b]
OMG	11.05 [5.97–15.44]	157.50 [50.34–572.51]
Onset age		
< 40	12.86 [8.49–19.46]	539.14 [427.19–710.41]
≥40	12.20 [6.74–19.69]	439.06 [74.13–608.83]
Thymoma		
With	23.20 [10.66–27.47][e]	669.82 [539.14–735.91][e]
Without	11.85 [7.60–14.62]	354.22 [69.84–544.83]

[b] $P < 0.05$ vs. OMG.
[e] $P < 0.01$ vs. Without.

between PD-1 on Tfh-cell membranes and PD-L1 on B-cell membranes; thus, Tfh cells would not receive strong enough negative regulatory signals in time. The relatively weak negative signals and the enhancement of positive signals would further promote Tfh-cell hyperfunction. Additionally, plasma sICOSL and sPD-1 levels were positively correlated with AchR-Ab concentrations in the USMG group, although there were no significant differences due to an insufficient sample size. The results suggest that sICOSL and sPD-1 affect the production of AchR Ab and participate in the pathological process of MG by regulating or interfering with Tfh-cell activation.

In recent years, the roles of soluble costimulatory molecules in disease stratification, disease assessment, and clinical prognosis have received increasing attention. In our study, sICOSL and sPD-1 levels were significantly increased in USMG-group patients with GMG compared to patients with OMG. The sICOSL and sPD-1 levels in the USMG group were both positively correlated with QMG scores. Moreover, these levels returned to normal during disease remission. These results indicated that sICOSL and sPD-1 have the potential for future use as biomarkers for the assessment of disease severity and drug-treatment response. Further studies to determine their threshold values are warranted.

In summary, this study showed that abnormal activation of the ICOS/ICOSL and PD-1/PD-L1 pathways participates in the immunopathological process of MG. The abnormal amplification of Tfh cells that results from the dysfunction and imbalance of these 2 groups of positive and negative costimulatory signals may be the critical pathogenic mechanism of MG. sICOSL and sPD-1 may be important factors affecting Tfh cells. Further studies targeting the specific mechanisms of soluble costimulatory molecules in MG immune disorders will provide new targets and directions for the treatment of MG.

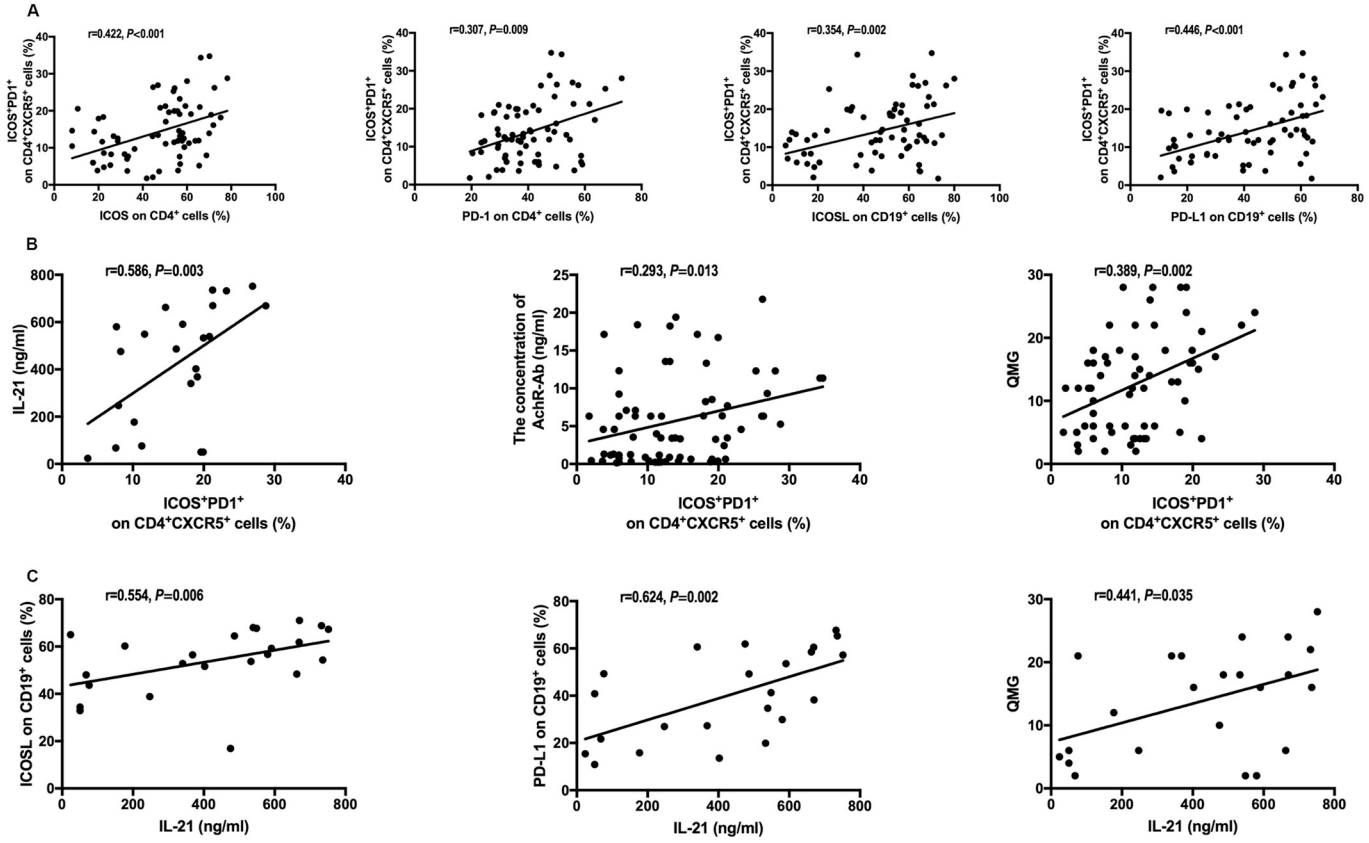

Fig. 5. Correlation between Tfh and laboratory and clinical parameters of the USMG group.

A. Linear correlations between the percentages of Tfh cells and ICOS/PD-1 expression on $CD4^+$ T cells and ICOSL/PD-L1 on $CD19^+$ B cells in the USMG group (n = 72).

B. Linear correlations between the percentages of Tfh cells and plasma IL-21 levels (n = 23), the concentration of AchR-Ab and QMG scores in the USMG group (n = 72).

C. Linear correlations between plasma IL-21 levels and ICOSL/PD-L1 expression on $CD19^+$ B cells and QMG scores in the USMG group (n = 23).

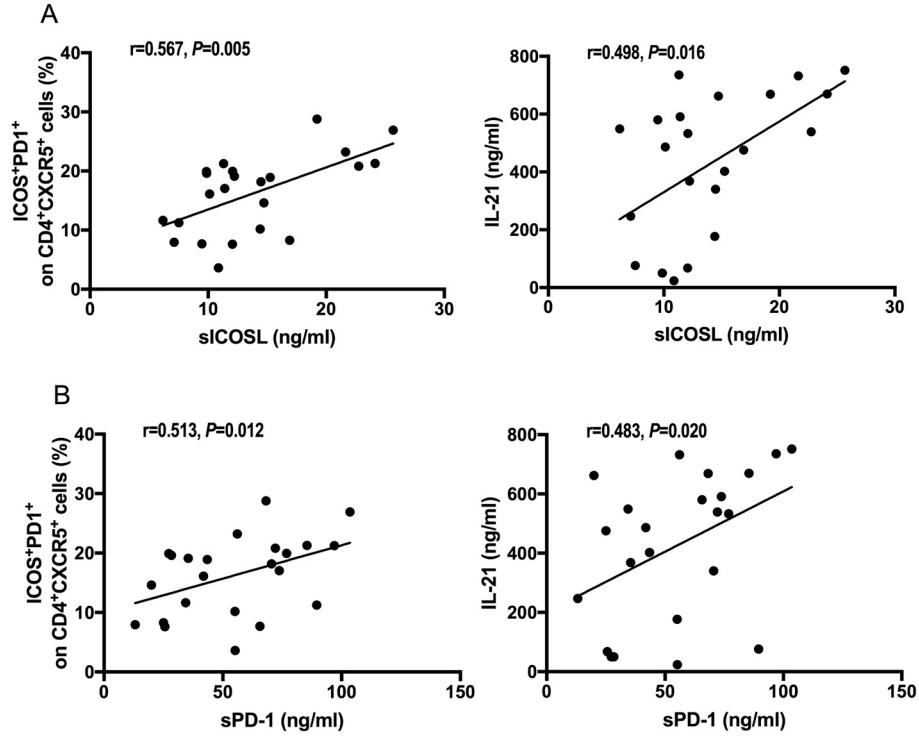

Fig. 6. Correlation between sICOSL and sPD-1 plasma levels and the percentages of Tfh cells and plasma IL-21 levels in the USMG group.

A. Linear correlation between sICOSL plasma levels and the percentages of Tfh cells and plasma IL-21 levels (n = 23).

B. Linear correlation between sPD-1 plasma levels and the percentages of Tfh cells and plasma IL-21 levels (n = 23).

Authors' contributions

X.Y., Y.G. participated in the study design, performed acquisition of data, statistical analysis, and drafted the manuscript. C.W., S.S., X.W., J.T. and M.W. performed the blood sample collection, processing and analysis by flow cytometry. X.J., X.D., H.G. Q.F. and W.D. contributed to the collection of clinical information of patients with myasthenia gravis. X.Z. instructed laboratory experiments. Q.X. designed the study, interpreted the data and revised the final manuscript to be published. All authors read and approved the final version.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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