European Review for Medical and Pharmacological Sciences 2023; 27: 10342-10364

TLR4-MyD88-NF-κB signaling imbalances Th17 and Treg cells in thymoma with myasthenia gravis

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Abstract. – OBJECTIVE: Thymus is an immune organ in which pathological changes may cause autoimmune diseases, including myasthenia gravis (MG). Recent studies have focused on Toll-like receptor 4 (TLR4) signaling as the cause of such changes. In our previous study, an imbalance of T helper 17 (Th17) cells and T regulatory (Treg) cells was found in MG thymoma. These results suggest the involvement of TLR4 in the pathogenesis of thymoma MG *via* an alteration of the Th17/Treg balance. Here, we aimed to assess whether the TLR4-MyD88-NF-κB pathway is upregulated in MG thymoma and its relationship with Th17/Treg cells.

PATIENTS AND METHODS: We collect thymoma samples from 54 patients with or without MG, detecting the expression level of TLR4, MyD88, and NF-κB in thymoma tissues. Next, we established an in vitro experiment of coculturing thymoma cells with CD4+ T cells and detected the differentiation of Th17 cells and Treg cells and their marker protein, retinoid-related orphan receptor gamma t (RORγt) and forkhead transcription factor 3 (Foxp3).

RESULTS: We found TLR4, MyD88, and NF-κB expressed more in MG thymoma compared with simple thymoma. After the transwell coculturing, we observed an imbalance of Th17/Treg cells after TLR4 stimulation.

CONCLUSIONS: TLR4 is stimulated in thymoma, causing an increase of Th17 cells and a decrease of Treg cells, namely an imbalance of Th17/Treg cells, resulting in MG.

Key Words:
TLR4, Thymoma, Th17, Treg, MG.

Introduction

$CD4^+$ T lymphocytes develop and mature in the thymus, and they will differentiate into various cell types, secreting distinct cytokines, including classical Th1, Th2, Tfh, and Th17, Treg cells. Among these cells, Th17 and Treg cells, which are respectively characterized by expressing transcription factor RORγt and Foxp3, coordinate to regulate the immune system and maintain the balance of autoimmunity by performing opposite roles in immune responses[1]. Th17 cells express proinflammatory cytokines interleukin (IL) 17, IL-22, and IL-26 that promote inflammation and autoimmunity[2,3], whereas Treg cells express anti-inflammatory cytokines IL-2, IL-10, and IL-35 that suppress inflammation and autoimmunity[4]. Therefore, an excess of Th17 cells or a lack of Treg cells, which is interpreted as an imbalance, causes diverse autoimmune diseases, including multiple sclerosis, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE)[5-9].

TLR4 is a type of membrane glycoprotein expressed on several immune cell types, which functions as an identifier to recognize bacteria, viruses, parasites, and other pathogens in innate immunity, and regulates Th17 and Treg cell numbers[10]. TLR4 also plays an essential role in the adaptive immune system. TLR4 stimulation enhances Th17 cell differentiation and their proinflammatory cytokine production and inhibits Treg cell induction and their suppressive function[11-13]. In general, TLR4 activation contributes to the initiation of autoimmune diseases by regulating the balance of Th17 and Treg cells.

Myasthenia gravis (MG) is a chronic autoimmune disease with a neuromuscular disorder, which has a close association with thymoma. In thymoma patients, because of an alteration in the microenvironment for T cell development, there may be a higher probability of the imbalance of Th17/Treg cells to occur in thymoma tissue[14]. This is consistent with studies indicating an imbalance of Th17 and Treg cells in MG patients[15,16]. Similarly, in our previous study, more Th17 cells accompanied by higher RORγt expression and few-

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er Treg cells followed by lower Foxp3 expression were found in MG thymoma tissue and peripheral blood than in simple thymoma tissue[17]. Additionally, in MG patients with thymitis and thymic involution, overexpression of TLR4 signaling has been observed relative to the healthy thymus[18]. Furthermore, TLR4 stimulation increases C-C motif chemokine ligand (CCL17) expression, augments Th17-related cytokine production, and upregulates Th17-related gene expression in myasthenic patients with thymitis and hyperplastic thymus[19]. However, the connection between the TLR4 signaling pathway and the imbalance of Th17 and Treg cells in MG thymoma is unclear.

Therefore, we evaluated the pathological effects of TLR4 signaling on MG thymoma by assessing the correlation between TLR4 signaling and Th17/Treg cell numbers. The effect of altered TLR4 signaling on cytokine secretion was also investigated in a thymoma cell line. Ultimately, to better demonstrate the effect of TLR4 signaling in the thymoma milieu on $CD4^+$ T cells differentiation, we established an *in vitro* model by coculturing $CD4^+$ T cells and thymoma cells in a transwell system. Our study aim is to investigate the potential pathogenesis of thymoma-associated MG.

Patients and Methods

Patient Recruitment

In accordance with our inclusion and exclusion criteria shown in Table I, 54 patients pathologically diagnosed with thymoma were recruited at Tianjin Medical University General Hospital from 2016 to 2020, of which 25 had simple thymoma without related autoimmune diseases, and 29 had thymoma and MG. Patients with other autoimmune diseases, receiving chemotherapy or radiotherapy were excluded. Preoperative peripheral blood was collected before immunotherapy and then anticoagulated. The serum was isolated for enzyme-linked immunosorbent assays (ELISAs) and stored at -80°C. Thymectomy was performed in all subjects and sufficient tissue samples were collected for further study. The ethics committee of Tianjin Medical University General Hospital approved this study, and informed consent was obtained from all subjects.

Immunohistochemistry of Thymic Sections

5-μm-thick paraffin-embedded sections of thymic tissues were heated at 60°C for 30 min, dewaxed in xylene, dehydrated in ethanol, and subjected to antigen retrieval by boiling in Tris-EDTA. An endogenous peroxidase blocker (SP-9000, ZSGB-bio, Beijing, China) was applied for 10 min. Non-specific reactions were blocked by incubation in goat serum for 10 min at room temperature. Sections were incubated with primary antibodies specific for IL-17 (1:250 dilution; ab79056, Abcam, Cambridge, UK), IL-6 (1:600 dilution; ab6672, Abcam, Cambridge, UK), TGF-β (1:100 dilution; ab215715, Abcam, Cambridge, UK), CCL17 (1:100 dilution; ab182793, Abcam, Cambridge, UK), CXCL13 (1:500 dilution; ab246518, Abcam, Cambridge, UK), TLR4 (1:50 dilution; ab13556, Abcam, Cambridge, UK), MyD88 (1:100 dilution; ab133739, Abcam, Cambridge, UK), or NF-κB P65 (1:300 dilution; ab16502, Abcam, Cambridge, UK) overnight at 4°C. Secondary labeling was performed with biotin-labeled goat anti-mouse/rabbit IgG polymer followed by incubation with horseradish peroxidase (HRP), diaminobenzidine (ZLI-9019, ZSGB-bio, Beijing, China), and hematoxylin (ZLI-9610, Origene, Wuxi, China). The images were captured digitally using a microscope (×200, Olympus, Tokyo, Japan) and their average optical density (AOD) was analyzed using ImageJ software (Wisconsin, USA).

Table I. Criteria for patients.

Inclusion criteria:	Exclusion criteria:
1) Age of 18-75, regardless of gender or ethnicity. 2) Patients with thymectomy. 3) Thymoma with MG or thymoma alone confirmed by pathology. 4) Pathological type: one of type A, type AB, type B1, type B2, type B3. 5) Patients with sufficient specimens for the experiment.	1) Those who did not meet the inclusion criteria. 2) Patients with SLE, RA and other autoimmune diseases. 3) Patients undergone preoperative radiotherapy, chemotherapy or other drugs targeting the tumor.

Immunofluorescence

Frozen 5-μm-thick thymic sections were heated at 60°C for 30 min, de-waxed in xylene, dehydrated in ethanol, and then subjected to antigen retrieval in Tris-EDTA at high temperature. Each section was incubated with primary antibodies specific for TLR4, MyD88, and NF-κB. Secondary labeling was performed by incubation in PANO Polymer HRP Ms+Rb (PNO70520AD, Panovue, Beijing, China), followed by staining with PANO reagent (PNO70520AD, Panovue, Beijing, China). Antigen retrieval and staining were repeated three times in sequence. Nuclei were counterstained with DAPI (PN200619AD, Panovue, Beijing, China). Sections were then sealed by PANO mounting medium (PN200319AD, Panovue, Beijing, China). Images were digitally acquired under the microscope (×200).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from thymoma samples and Thy0517 cells using Trizol reagent (9108, Takara, Dalian, China). Then, total RNA was reverse transcribed into cDNA using Oligo DT (3806, Takara, Dalian, China), reverse transcriptase (AI20926A, Takara, Dalian, China) and dNTP (4019, Takara, Dalian, China) in a thermal cycler (BioRad, Shanghai, China). cDNA was amplified with SYBR Premix Ex Taq™ (R001B, Takara) and corresponding primers (Sangon Biotech, Shanghai, China). Primer sequences for *TLR4*, *MyD88*, *NF-κB*, *IL-6*, *TGF-β*, *IL-17*, *CCL17*, *CXCL13*, and *GAPDH* were designed by GeneRunner (Table i). Amplification was performed at 95°C for 1 min for predenaturation, followed by 40 cycles of 95°C for 5 s for denaturation and 60°C for 30 s for annealing and extension. Relative expression levels of target genes were determined using the $2^{-\Delta\Delta Cq}$ method.

Cell Line and Culture

The thymoma cell line Thy0517 provided by the Cardiothoracic Surgery Department of Tianjin Medical University General Hospital (patent number: ZL 2014 1 0312866.6) was established from a type AB thymoma with myasthenia gravis patient. Cells were cultured in high glucose Dulbecco's modified Eagle's medium (C11995500BT, Thermo Fisher, Beijing, China) with 10% heat-inactivated fetal bovine serum (NQBB, Victoria, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO_2 incubator (Thermo Fisher, Waltham, CA, USA) at 37°C.

2 ml Cells were seeded in a 6-well plate, at 1×10^6/ml per well and treated with various concentrations of lipopolysaccharide (LPS) (0.01, 0.1, 1, and 10 μg/ml) and TAK (Butanamide, N-[2-[4-[[3-chloro-4-[3-(trifluoromethyl)phenoxy]phenyl]amino]-5H-pyrrolo[3,2-d]pyrimidin-5-yl]ethyl]-3-hydroxy-3-methyl)) (0.01, 0.1, 1, and 10 μg/ml). After culturing for 24, 48, and 72 h, cells were collected for RT-PCR analysis to measure the expression of TLR4, MyD88, and NF-κB and determine optimal concentrations (10 μg/ml for LPS and 1 μg/ml for TAK).

ELISAs

After culturing for 24, 48, and 72 h, the culture supernatant was collected and centrifuged for 10 min (150 g) to remove cell debris. IL-6, IL-17, CCL17, CXCL13, and TGF-β concentrations were quantified using commercially available ELISA kits (Proteintech Group Inc, Chicago, IL, USA) in accordance with the manufacturer's instructions.

Isolation of $CD4^+$ T Cells

PBMCs were separated from the fresh blood of normal volunteers using Ficoll (LTS1077, TBDsciences, Tianjin, China). Total $CD4^+$ cells were positively selected from PBMCs by magnetic separation (Human $CD4^+$ microbeads, Miltenyi Co., Ltd. Shanghai, China) in accordance with the manufacturer's instructions.

Coculture of $CD4^+$ T and Thy0517 Cells

Thy0517 cells were seeded in the lower chambers of 6-well transwell plates (3 mm pore size, Corning Costar) and treated with 10 μg/ml LPS or TAK $CD4^+$. T cells and DCs were seeded in the upper chambers. After 24 and 48 h of culture, $CD4^+$ T cells were collected for western blot analysis and flow cytometry. This experiment included the following groups. Group A: $CD4^+$ T and Thy0517 cell coculture; Group B: $CD4^+$ T and Thy0517 cell coculture treated with LPS; Group C: $CD4^+$ T and Thy0517 cell coculture treated with TAK.

Western Blotting

Cells were collected and ultrasonically homogenized in RIPA buffer. The protein concertation was then quantified by a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China). Protein samples were separated by electrophoresis in sodium dodecyl sulfate (SDS) -denaturing 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked

in PBS containing 5% BSA, incubated overnight at 4°C with primary antibodies specific for Foxp3 (1:4,000 dilution; ab20034, Abcam, Cambridge, UK) and RORγt (1:4,000 dilution; 13205-1-AP, Proteintech, Wuhan, Chian), and then incubated with IRDye-conjugated secondary antibodies (1:5,000 dilution; SA00001-1/SA00001-2, Proteintech, Wuhan, Chian). Membranes were scanned using an Odyssey Infrared Imaging System Scanner (Tanon, Shanghai, Beijing). Images were analyzed using ImageJ software. The relative expression of target proteins was determined using the $2^{-\Delta\Delta Cq}$ method.

Flow Cytometry

Cells were resuspended in 1 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Inc, CA, USA), and then 2 μl (this unit is correct) cell stimulation cocktail (eBioscience Inc, CA, USA) was added, followed by incubation for 5 h at 37**°C** with 5% CO_2. Cells were surface labeled with antibodies against each subpopulation for 20 min at room temperature. Following fixation and permeabilization, intracellular staining of FOXP3 and IL-17A was performed. For Th17 cell analysis, cells were stained with PerCP-cy5.5-conjugated anti-human CD3 (551163, BD Biosciences, San Jose, CA, USA), APC-conjugated anti-human CD8 (561421, BD Biosciences, San Jose, CA, USA) and PE-conjugated anti-human IL-17A (559502, BD Biosciences, San Jose, CA, USA) antibodies. For Treg cell analysis, cells were stained with FITC-conjugated anti-human CD4 (556615, BD Biosciences, San Jose, CA, USA), APC-conjugated anti-human CD25 (558643, BD Biosciences, San Jose, CA, USA), and PE-conjugated anti-human FOXP3 (563101, BD Biosciences, San Jose, CA, USA) antibodies. Isotype controls were used for compensation and to confirm antibody specificity. Stained cells were analyzed by a (FACSCanto II, BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo software.

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical analysis was conducted using Student's *t*-test for paired samples, single-factor (one-way/two-way) analysis of variance for multi-group data, and the Chi-squared test for categorical data. $p<0.05$ was considered statistically significant. Data were processed using SPSS 19.0 (IBM Corp., Armonk, NY, USA).

Results

Patient Characteristics

A total of 54 patients were enrolled in this study, including 25 patients with simple thymoma (Tm group) and 29 patients with thymoma and MG (MG group). There was no significant difference between the two groups regarding sex, mean age, and World Health Organization (WHO) histological classification of thymoma. Patient clinical characteristics are summarized in Table II.

Table II. Primer sequences.

Gene		Primer sequences
TLR4	sense	5'-TGAGCAGTCGTGCTGGTATC-3'
	Anti-sense	5'-CAGGGCTTTTCTGAGTCGTC-3'
MyD88	sense	5'-GCACATGGGCACATACAGAC-3'
	Anti-sense	5'-TGGGTCCTTTCCAGAGTTTG-3'
NF-κB	sense	5'-CTGAACCAGGGCATACCTGT-3'
	Anti-sense	5'-GAGAAGTCCATGTCCGCAAT-3'
IL-6	sense	5'-AGGGCTCTTCGGCAAATGTA-3'
	Anti-sense	5'-GAAGGAATGCCCATTAACAACAA-3'
TGF-β	sense	5'-GCTCCACGGAGAA-GAACAGGCTG-3'
	Anti-sense	5'-CTGCTCCAC-CTTGGGCTTGC-3'
IL-17	sense	5'-GGAACGTGGACTACCACATG-3'
	Anti-sense	5'-GCGCAGGACCAGGATCTCT-3'
CCL17	sense	5'-AGACATCTGAGGACTGCT-3'
	Anti-sense	5'-GTGAGGAGGCTTCAAGAC-3'
CXCL13	sense	5'-CGACATCTCTGCTTTCAT-3'
	Anti-sense	5'-TCTCTTGGACACATCTACAC-3'

Expression of TLR4, MyD88, and NF-κB in Thymoma

Immunohistochemical staining was used to measure protein expression, defined as the percentage of the visual field area occupied by positive expression in thymoma tissues. The results showed an increased distribution of TLR4, MyD88, and NF-κB (P65) in the MG group compared with that in the Tm group (Figures 1-3). As shown in Figure 4A, TLR4 expression in the MG

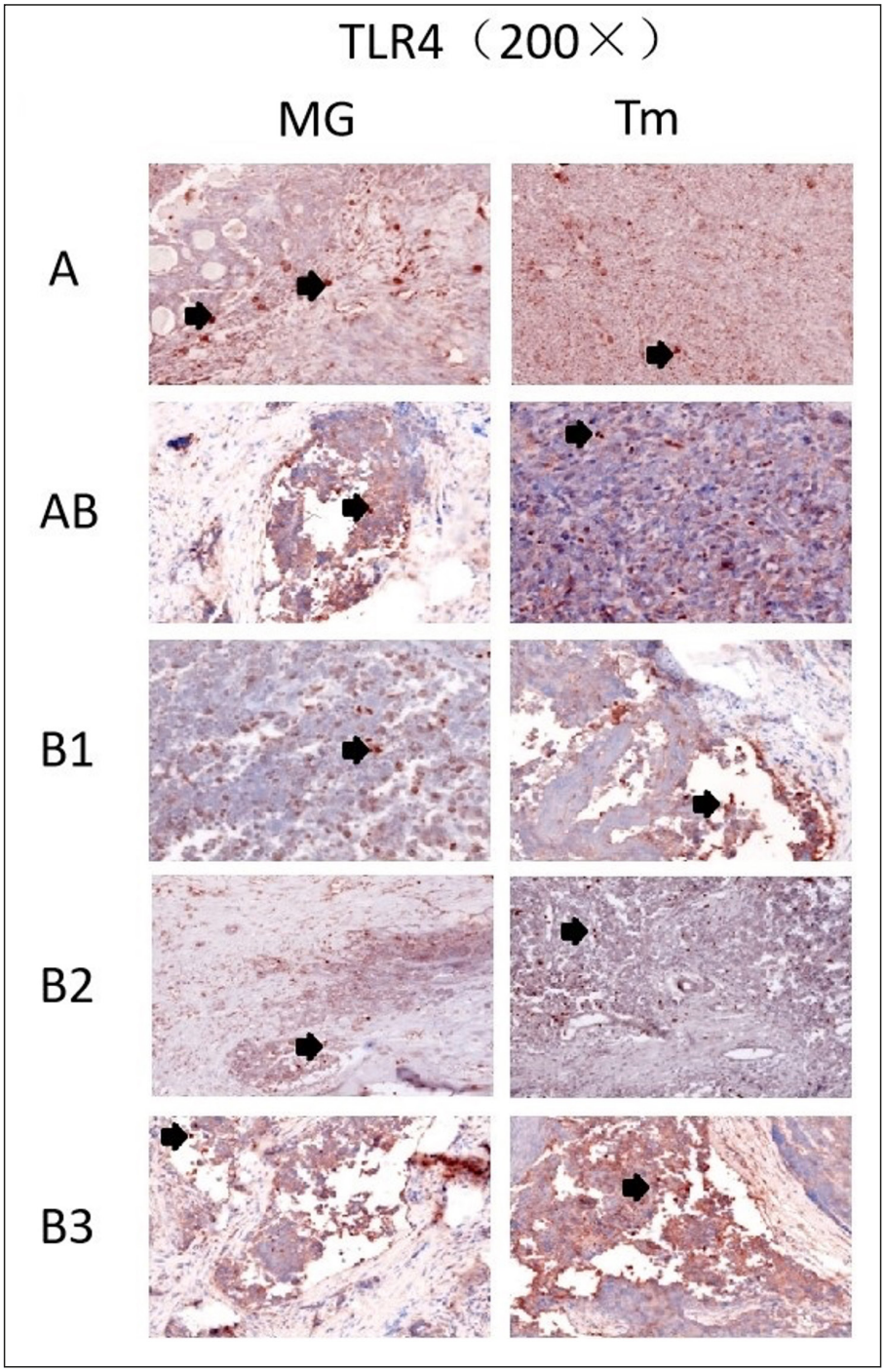


Figure 1. Expression of TLR4. Immunohistochemical staining of TLR4 in five histological types (**A**, **AB**, **B1**, **B2**, **B3**) of thymoma in MG and Tm groups (×200), showing protein expression (brown) calculated by the AOD (average optical density) of the area of positive expression.

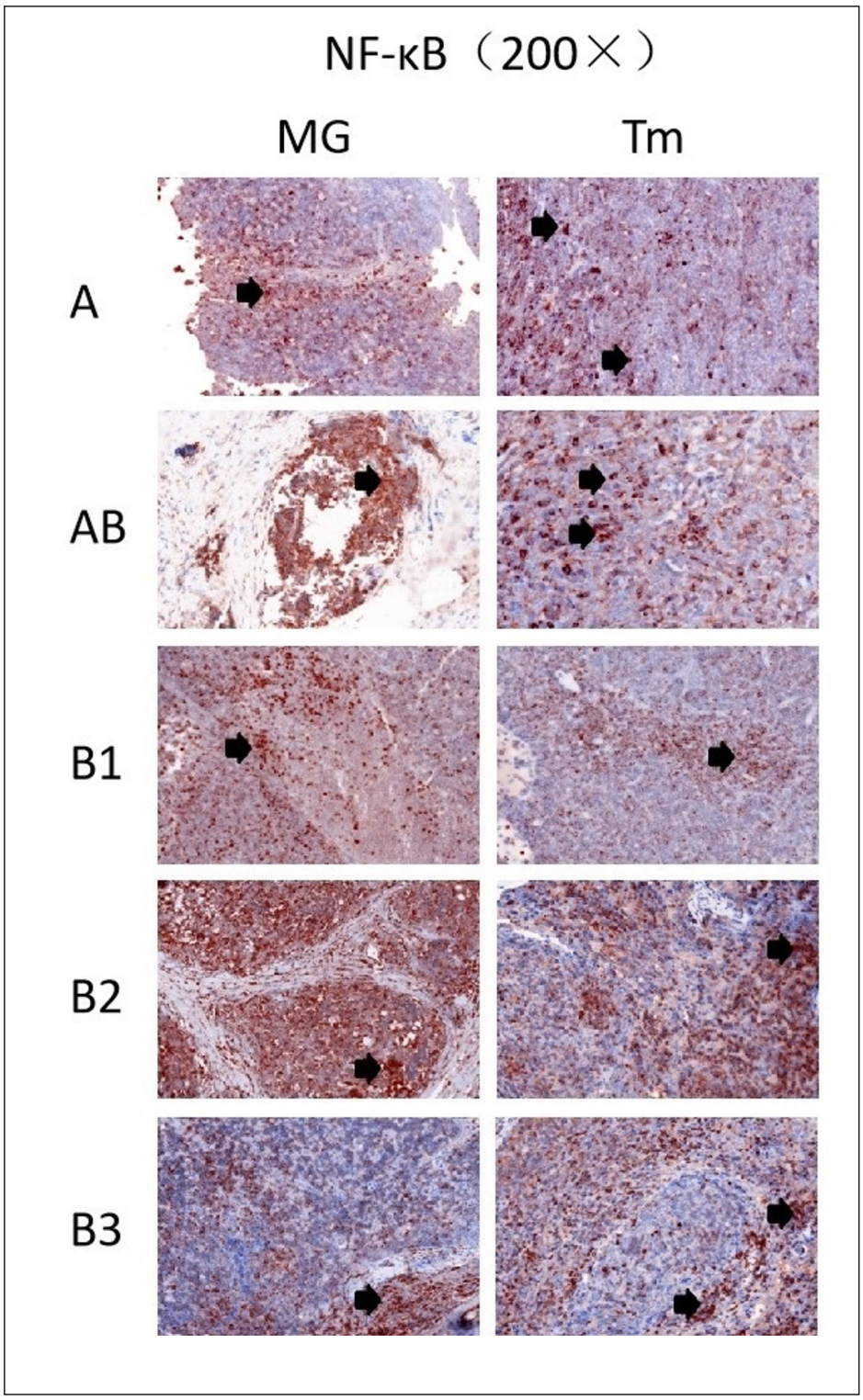


Figure 2. Expression of MyD88. Immunohistochemical staining of MyD88 in five histological types (**A**, **AB**, **B1**, **B2**, **B3**) of thymoma in MG and Tm groups (×200), showing protein expression (brown) calculated by the AOD of the area of positive expression.

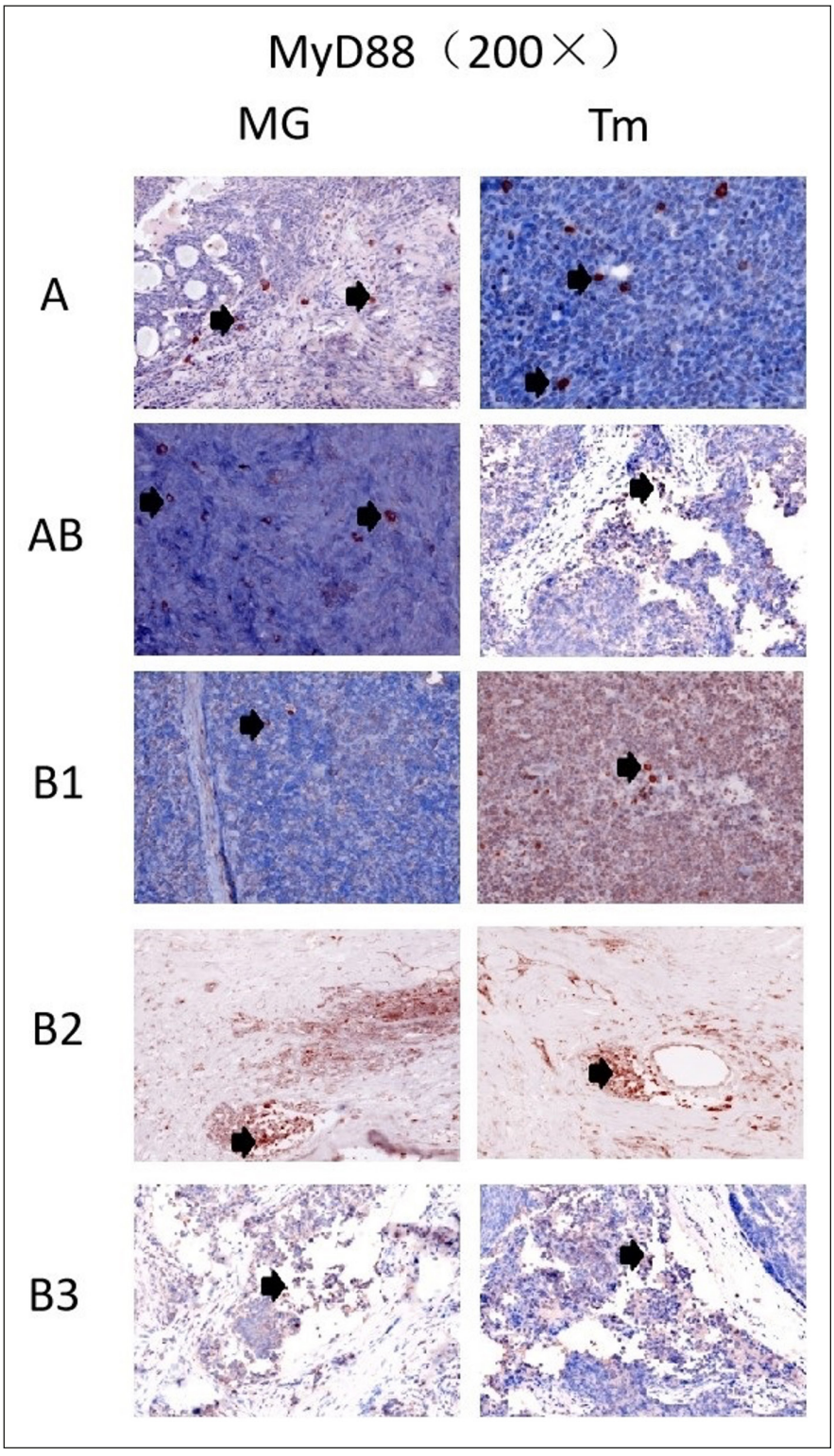


Figure 3. Expression of NF-κB (P65). Immunohistochemical staining of NF-κB (P65) in five histological types (**A**, **AB**, **B1**, **B2**, **B3**) of thymoma in MG and Tm groups (×200), showing protein expression (brown) calculated by the AOD of the area of positive expression.

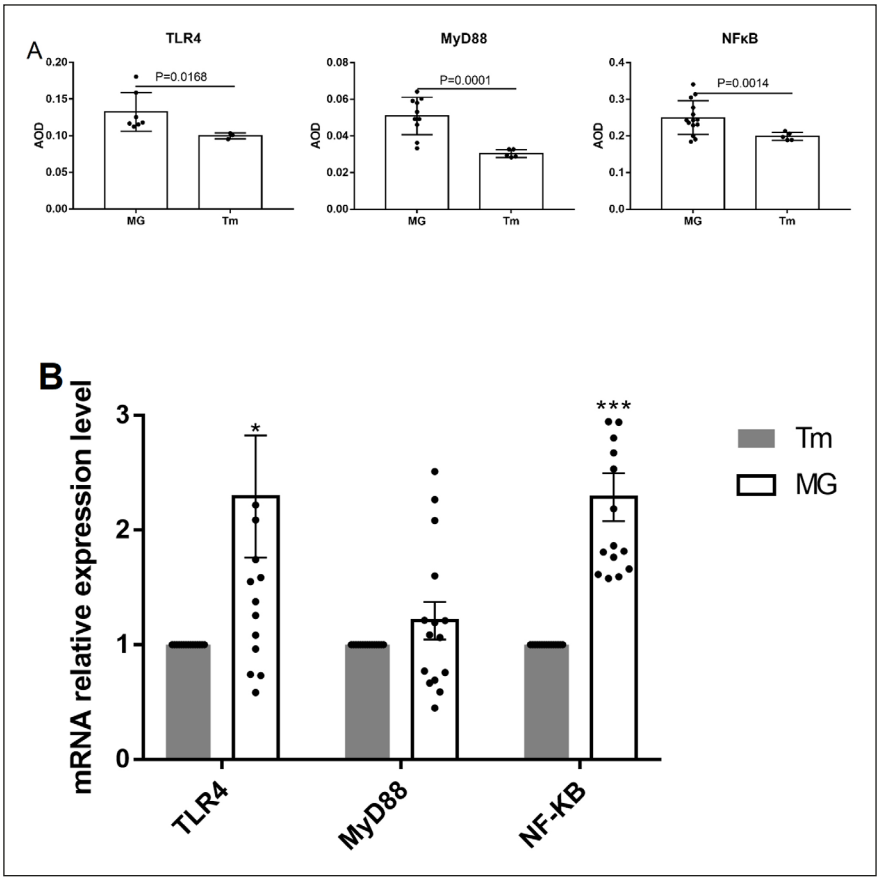


Figure 4. Expression of TLR4, MyD88, and NF-κB (P65). **A**, Protein expression levels of TLR4, MyD88, and NF-κB (P65) in thymomas of MG and Tm groups. **B**, Relative mRNA expression of TLR4, MyD88, and NF-κB (P65) in patients with MG thymoma compared with patients with simple thymoma. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

group (0.132±0.026) was higher than that in the Tm group (0.100±0.004) (p=0.0168), and MyD88 expression in the MG group (0.051±0.010) was higher than that in the Tm group (0.030±0.002) (p=0.0001). Similarly, NF-κB (P65) expression in the MG group (0.250±0.046) was higher than that in the Tm group (0.199±0.011) (p=0.0014) (Figure 4B, Table IV). We further examined the expression of TLR4, MyD88, and NF-κB (P65) in MG thymoma by polychromatic immunofluorescence staining and observed colocalization of TLR4, MyD88, and NF-Kb (P65) (Figure 5).

RT-qPCR was applied to measure the mRNA levels of TLR4, MyD88, and NF-κB (P65). The result was calculated as the relative expression level in the MG group to the Tm group. As shown

Table III. Patient characteristics.

	Thymic histology	MG group (n=29)	Tm group (n=25)	p-value
sex (M/F)	A	2/1	1/1	1.00
	AB	3/2	2/3	1.00
	B1	5/3	4/2	1.00
	B2	3/2	4/1	1.00
	B3	2/6	5/2	0.13
age (years, mean±SD)	A	52.67±18.72	61.50±2.12	0.57
	AB	52.60±3.13	55.00±15.10	0.74
	B1	43.25±14.69	56.17±6.27	**0.05**
	B2	47.00±11.22	52.60±10.55	0.44
	B3	56.75±13.93	48.29±13.17	0.25

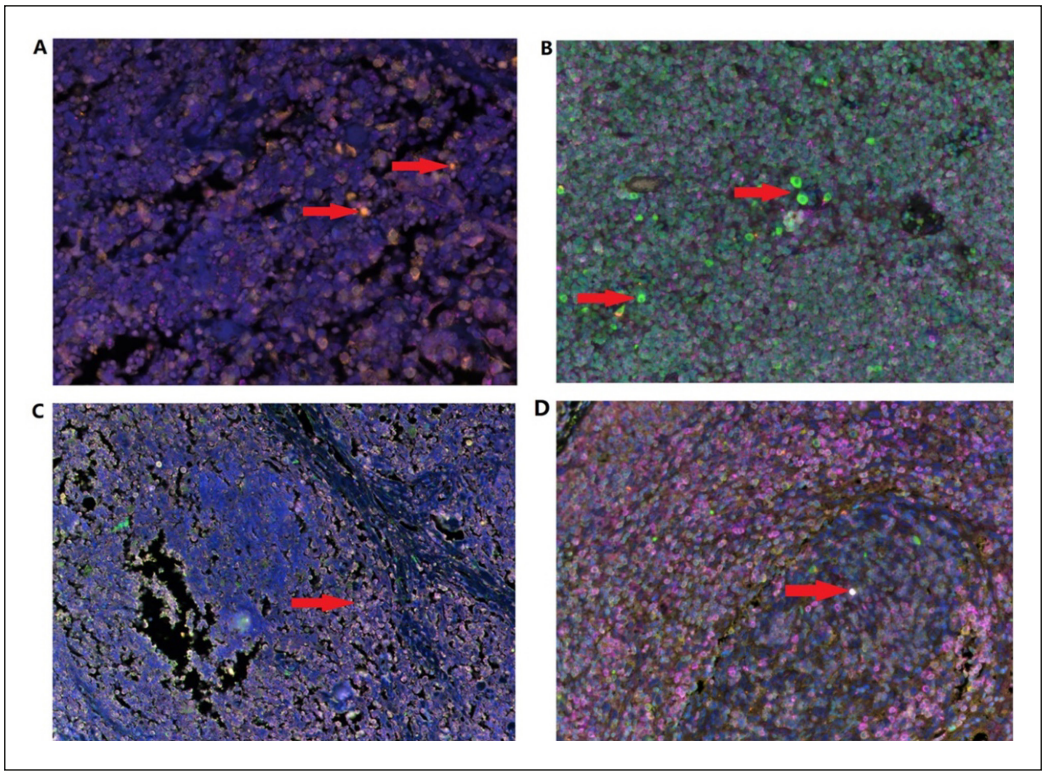


Figure 5. Polychromatic immunofluorescence staining of TLR4, MyD88, and NF-κB (P65) in thymoma. **A**, Orange area indicated by the red arrow represents TLR4. **B**, Green area indicated by the red arrow represents MyD88. **C**, Pink area indicated by the red arrow represents NF-κB (P65). **D**, White area indicated by the red arrow represents the colocalization of TLR4, MyD88, and NF-κB (P65).

in Figure 1E, TLR4 (2.294±2.060) (p=0.029) and NF-κB (P65) (2.288±0.808) (p<0.001) mRNAs were increased in the MG group compared with the Tm group and MyD88 showed no significance difference (p=0.222) (Figure 4B, Table V).

Expression of IL-6, TGF-β, IL-17, CCL17, and CXCL13 in Thymoma Tissues

Next, we examined IL-6, TGF-β, IL-17, CCL17, and CXCL13 in thymoma tissues (Figures 6-8). IL-6 expression in the MG group (0.051±0.027) was higher than that in the Tm group (0.028±0.022) (p=0.002), TGF-β expression in the MG group (0.280±0.037) was higher than that in the Tm group (0.157±0.044) (p<0.001). IL-17 expression in the MG group (0.066±0.035) was higher than that in the Tm group (0.040±0.016) (p=0.001). CCL17 expression in the MG group (0.025±0.029) was higher than that in the Tm group (0.003±0.007) (p<0.001), and CXCL13 expression showed no significant difference between MG and Tm groups (p=0.116) (Figure 9A, Table IV).

Table IV. Protein levels of TLR4, MyD88, NF-κB, IL-6, TGF-β, IL-17, CCL17 and CXCL13 between MG group and Tm group.

Protein	MG group	Tm group	*p*-value
TLR4	2.70±4.97	0.42±1.37	**0.047**
MyD88	2.31±2.84	0.98±2.50	**0.018**
NF-κB	9.30±7.44	3.69±8.15	**0.011**
IL-6	0.051±0.027	0.028±0.022	**0.002**
IL-17	0.066±0.035	0.040±0.016	**0.001**
CCL17	0.0249±0.0288	0.0031±0.0066	**<0.001**
CXCL13	0.0035±0.0046	0.0016±0.0048	0.116

Table V. mRNA relative levels of TLR4, MyD88, NF-κB, IL-6, TGF-β, IL-17, CCL17 and CXCL13 between MG group and Tm group.

mRNA	Tm group	MG group	*p*-value
TLR4	1	2.29±2.06	**0.029**
MyD88	1	1.21±0.64	0.222
NF-κB	1	2.29±0.81	**<0.001**
IL-6	1	3.14±1.08	**<0.001**
IL-17	1	3.48±1.94	**<0.001**
CCL17	1	8.84±9.68	**0.017**
CXCL13	1	11.36±9.85	**0.006**

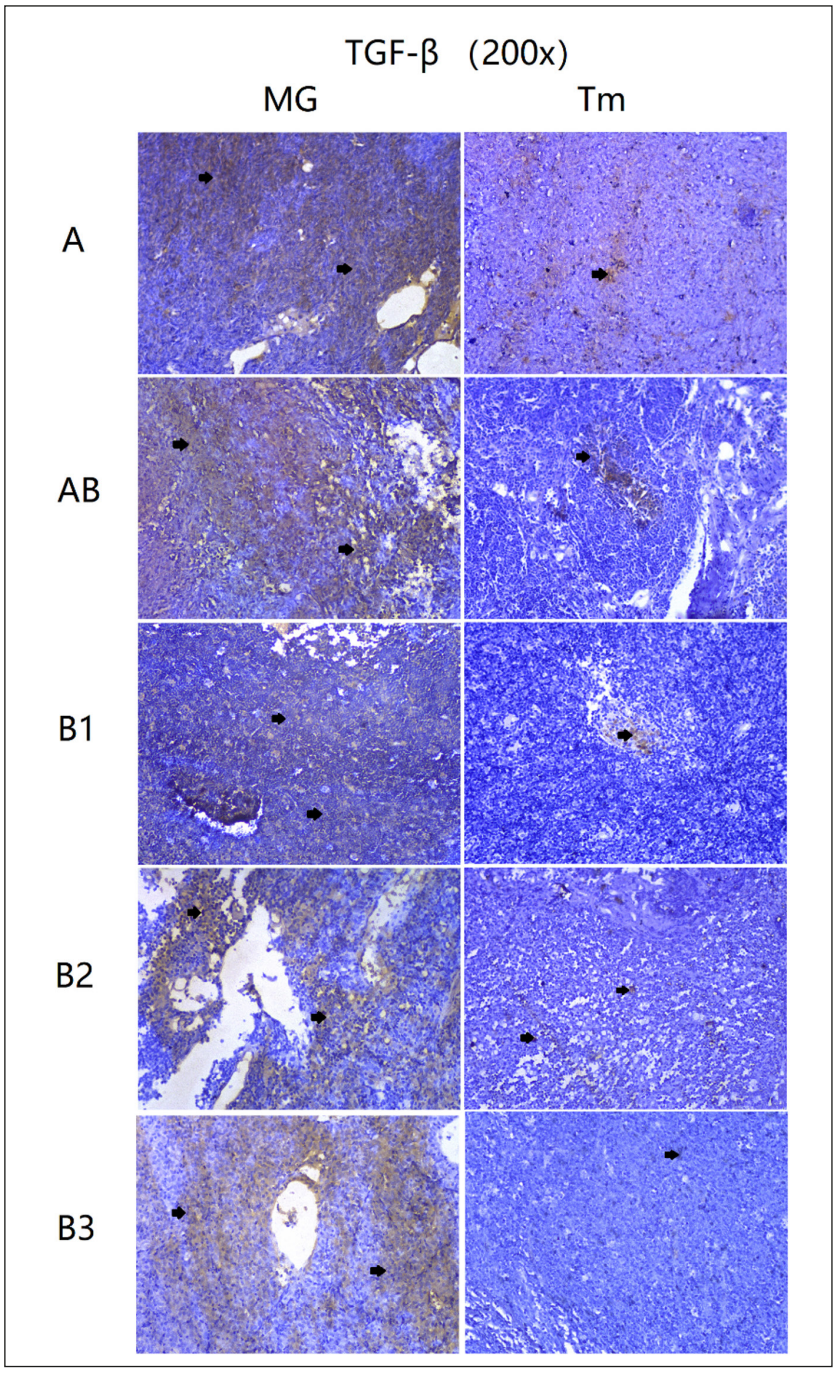


Figure 6. Expression of TGF-β. Immunohistochemical staining of TGF-β in five histological types (A, **AB**, **B1**, **B2**, **B3**) of thymoma in MG and Tm groups (×200), showing protein expression (brown) calculated by the AOD of the area of positive expression.

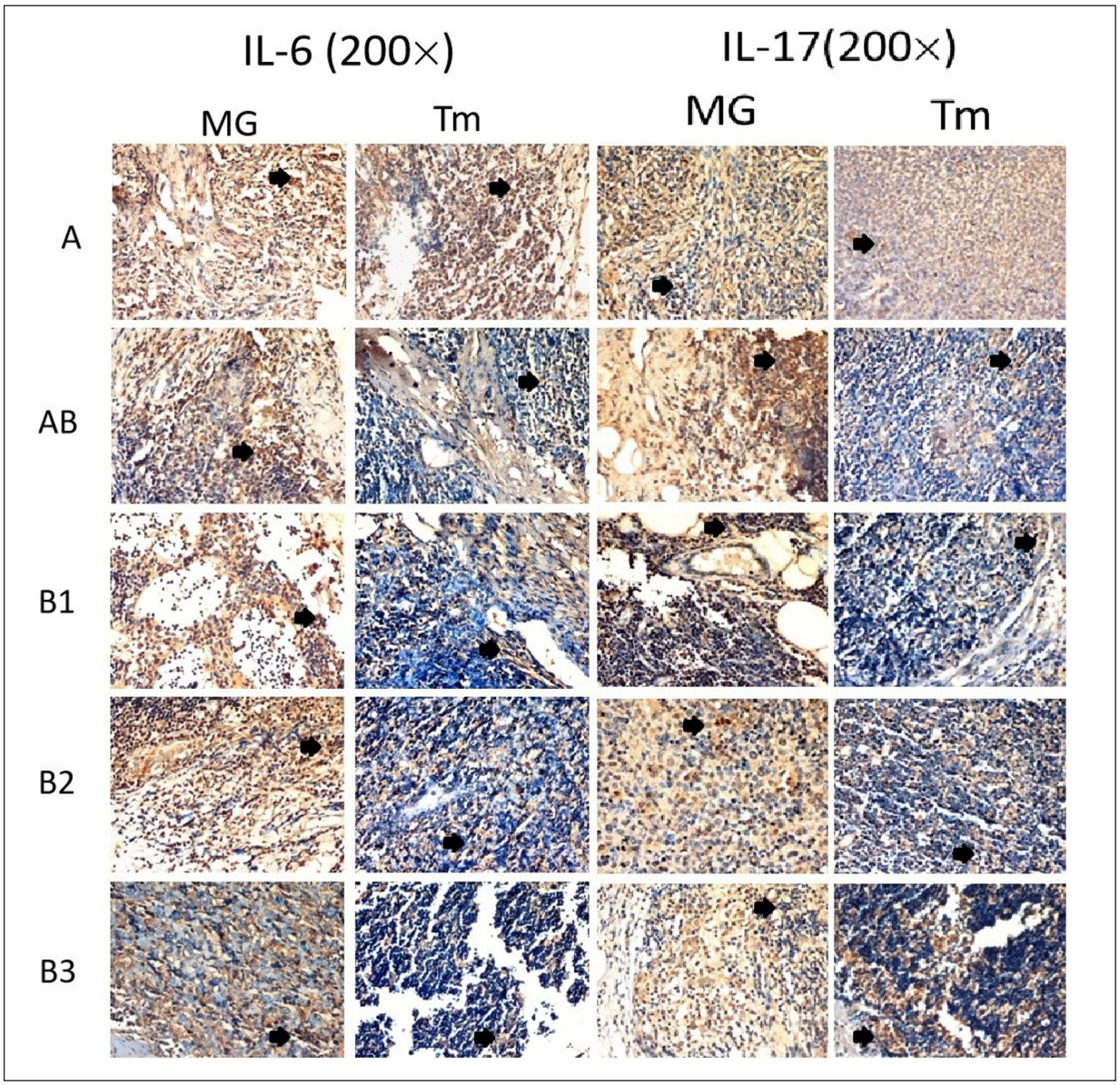


Figure 7. Expression of IL-6 and IL-17. Immunohistochemical staining of IL-6 and IL-17 in five histological types (**A**, **AB**, **B1**, **B2**, **B3**) of thymoma in MG group and Tm groups (×200), showing protein expression (brown) calculated by the AOD of the area of positive expression.

Relative mRNA expression of IL-6 (3.142±1.081) (p<0.001), TGF-β (9.45±11.99) (p=0.042), IL-17 (3.467±1.937) (p<0.001), CCL17 (8.841±9.677) (p=0.017), and CXCL13 (11.36±9.852) (p=0.006) was augmented in the MG group compared with the Tm group (Figure 9B, Table V).

IL-6, IL-17, CCL17, CXCL13, and TGF-β in Sera of Thymoma Patients

The concentrations of cytokines IL-6, IL-17, and TGF-β and chemokines CCL17 and CXCL13 were determined in sera from thymoma. TGF-β showed a significant difference between the MG group (45.63±5.36) and Tm group (35.20±5.85) (p <0.001), whereas IL-6, IL-17, CCL17, and CXCL13 showed no significant difference between MG and Tm groups (Figure 10, Table VI).

Stimulation And Inhibition of the TLR4 Signaling Pathway in Thy0517 Cells

To understand the pathological role of the TLR4 signaling pathway in thymoma, we determined whether TLR4 signaling affected the expression

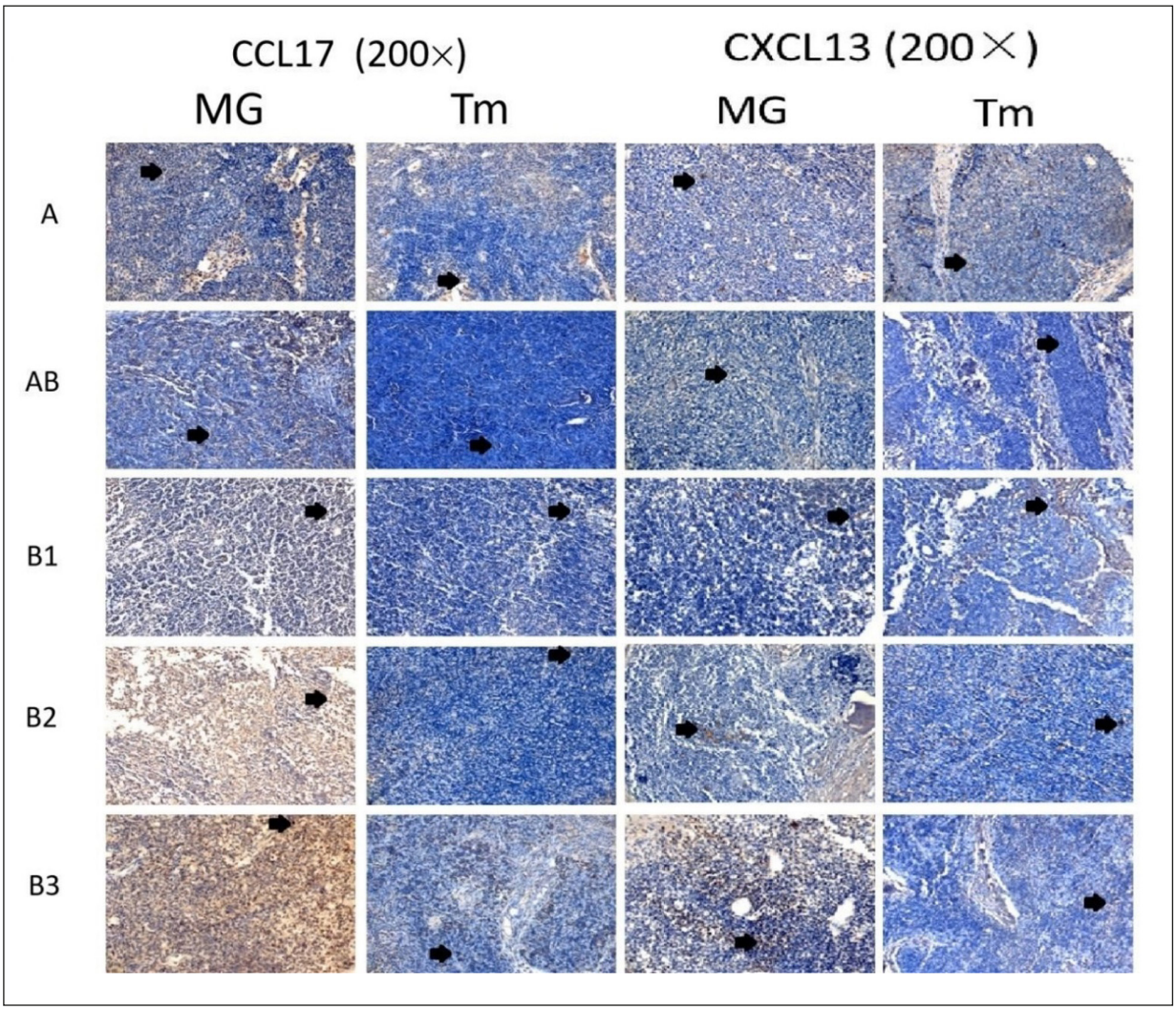


Figure 8. Expression of CCL17 and CXCL13. Immunohistochemical staining of CCL17 and CXCL13 in five histological types (**A**, **AB**, **B1**, **B2**, **B3**) of thymoma in MG and Tm groups (×200), showing protein expression (brown) calculated by the AOD of the area of positive expression.

of related cytokines and chemokines. We found that LPS stimulation enhanced TLR4 signaling in Thy0517 cells. After LPS stimulation for 24, 48, and 72 h, TLR4 mRNA was analyzed by RT-qPCR. Compared with the control group, TLR4 expression was significantly increased at 24 hours (5.082±1.374) (p<0.001), MyD88 expression was significantly increased at 48 hours (24.610±8.493) (p<0.001), and NF-κB expression was significantly increased at 48 hours (2.865±1.514) (p=0.047) (Figure 11, Table VII).

TLR4 signaling was blocked by the specific antagonist TAK. Compared with the control group, TLR4 expression was significantly reduced at 24 h (0.003±0.001) (p<0.001), 48 h (0.001±0.0004) (p<0.001), and 72 h (0.025±0.040) (p<0.001), MyD88 expression was significantly increased at 24 h (0.088±0.081) (p<0.001), 48 h (0.077±0.067) (p<0.001), and 72 h (0.222±0.251) (p<0.001), and NF-κB expression was significantly increased at 24 h (0.051±0.061) (p=0.005), 48 h (0.189±0.263) (p=0.011) and 72 h (0.313±0.537) (p=0.023) (Figure 11, Table VIII).

Expression of IL-6, IL-17, TGF-β, CCL17, and CXCL13 in Thy0517 Cells After Stimulating or Inhibiting TLR4 Signaling

Culture supernatants were collected for ELISAs after LPS treatment (Table IV). The level of IL-6 was significantly increased at 72 h (p<0.0001), and TGF-β was significantly decreased at 72 h (p<0.0001). IL-17, CCL17, and CXCL13 showed no significant changes (Figure 12A), but CCL17 showed a significant difference

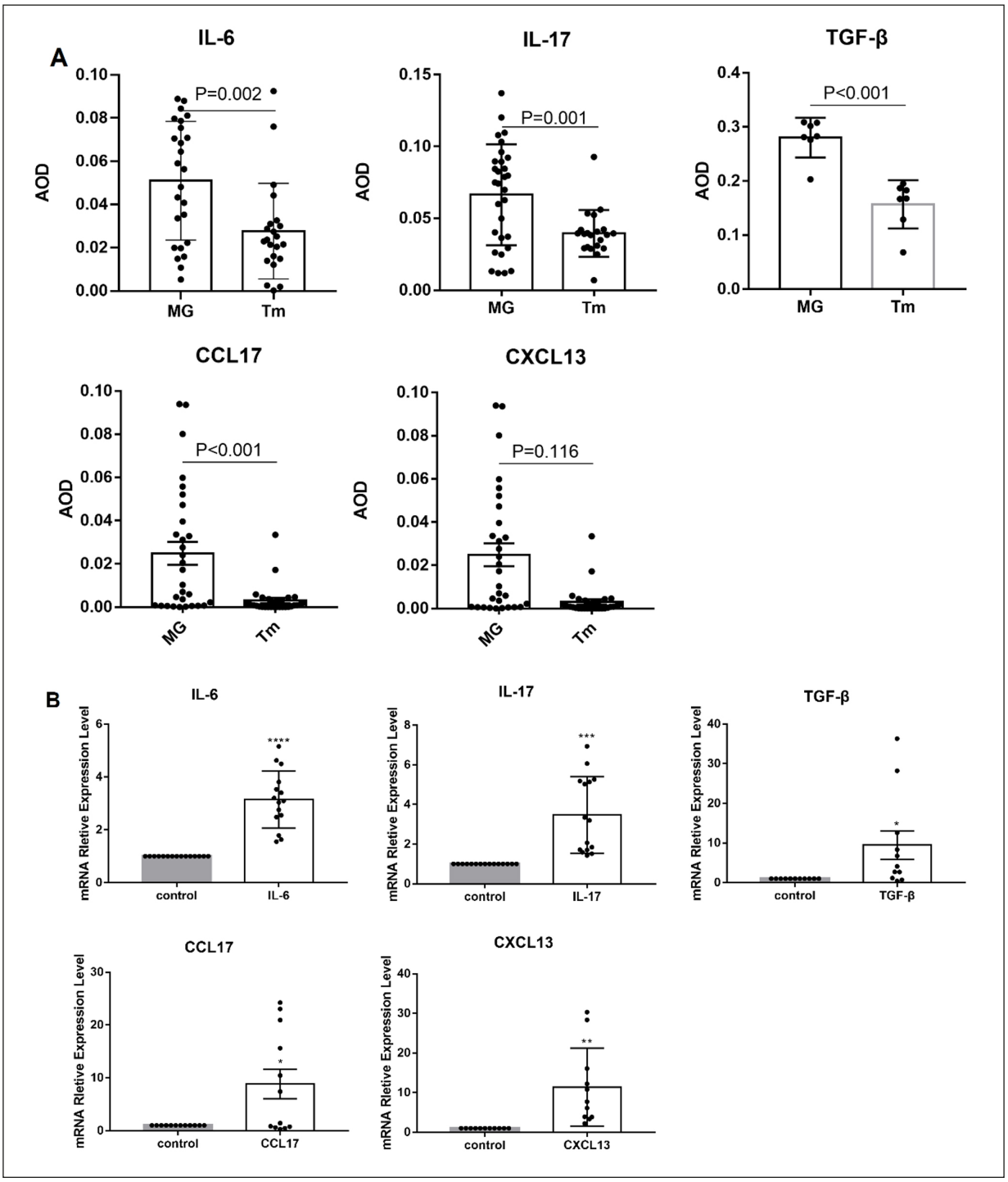


Figure 9. Expression of IL-6, TGF-β, and IL-17, CCL17, and CXCL13. **A**, Expression levels of IL-6, TGF-β, IL-17, CCL17, and CXCL13 in thymomas of MG and Tm groups. **B**, Relative mRNA expression of IL-6, TGF-β, IL-17, CCL17 and CXCL13 in patients with MG thymoma compared with patients with simple thymoma. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

among the four groups (p=0.045) (Table IX). After TAK treatment, IL-6 expression was significantly reduced at 24 h (p=0.007) and 48 h (p=0.016), and TGF-β expression was significantly increased at 48 h (p<0.0001) (Figure 12B). The remaining cytokines showed no significant alterations, while CCL17 showed a significant difference among the four groups (p=0.047) (Table X).

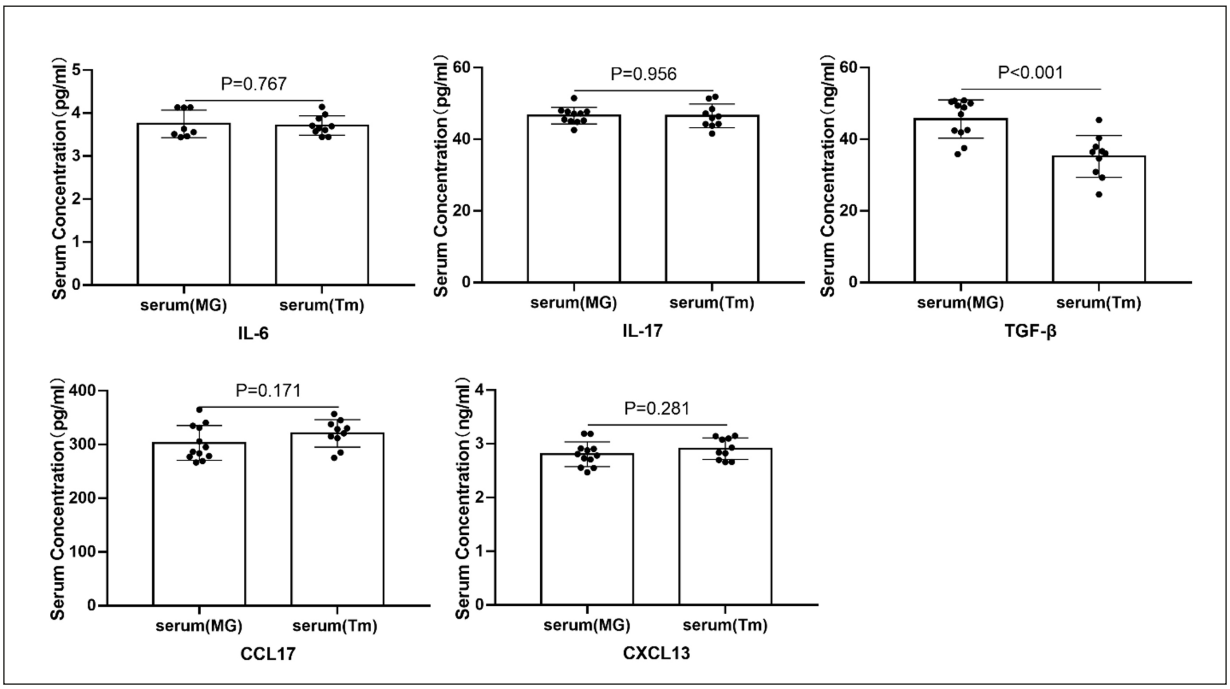


Figure 10. IL-6, IL-17, CCL17, CXCL13, and TGF-β in serum. IL-6, IL-17, CCL17, CXCL13, and TGF-β in sera from MG thymoma patients and simple thymoma patients. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table VI. The level of IL-6, IL-17, CCL17, CXCL13, and TGF-β in serum.

Target factors	MG group	Tm group	*p*-value
TGF-β (ng/ml)	45.63±5.36	35.20±5.85	**<0.001**
IL-6 (pg/ml)	3.75±0.32	3.71±0.23	0.767
IL-17 (pg/ml)	46.57±2.31	46.50±3.31	0.956
CCL17 (pg/ml)	302.81±32.35	320.71±25.38	0.171
CXCL13 (pg/ml)	2.81±0.23	2.91±0.20	0.281

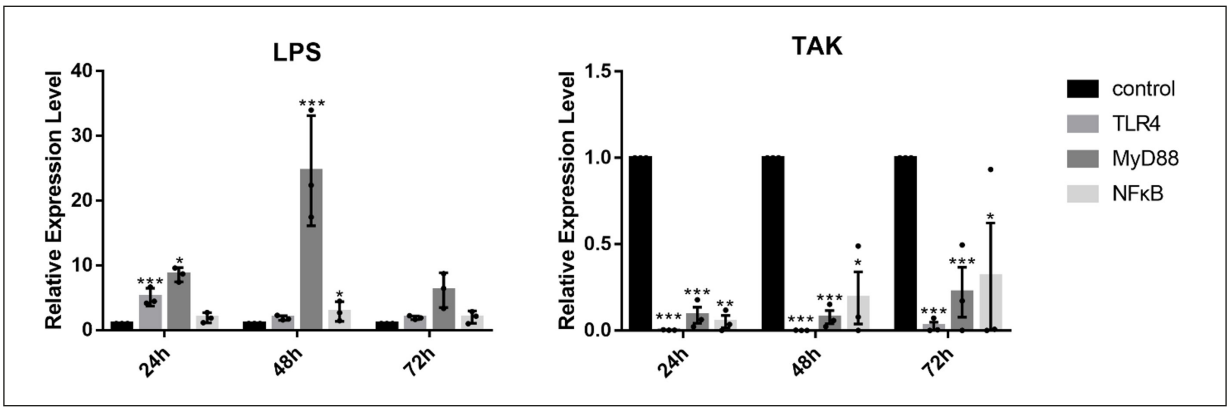


Figure 11. Expression of TLR4, MyD88, NF-κB (P65) after TLR4 regulation. Relative mRNA expression of TLR4, MyD88, NF-κB (P65) in Thy0517 cells after TLR4 regulation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table VII. mRNA relative levels of TLR4, MyD88, and NF-κB after LPS treatment.

mRNAs	Time	Control	LPS group	*p*-value
TLR4	24 h	1	5.082±1.374	**<0.001**
	48 h	1	1.867±0.365	0.181
	72 h	1	1.907±0.281	0.164
MyD88	24 h	1	8.550±1.095	0.073
	48 h	1	24.610±8.493	**<0.001**
	72 h	1	6.175±2.695	0.196
NFκB	24 h	1	1.901±0.771	0.290
	48 h	1	2.865±1.514	**0.047**
	72 h	1	1.985±0.951	0.250

Table VIII. mRNA relative levels of TLR4, MyD88, and NF-κB after TAK treatment.

mRNAs	Time	Control	TAK group	*p*-value
TLR4	24 h	1	0.003±0.001	**<0.001**
	48 h	1	0.001±0.0004	**<0.001**
	72 h	1	0.025±0.040	**<0.001**
MyD88	24 h	1	0.088±0.081	**<0.001**
	48 h	1	0.077±0.067	**<0.001**
	72 h	1	0.222±0.251	**<0.001**
NFκB	24 h	1	0.051±0.061	**0.005**
	48 h	1	0.189±0.263	**0.011**
	72 h	1	0.313±0.537	**0.023**

Th17 and Treg Cells Among CD4⁺ T Cells Cocultured with Thy0517 Cells

To assess the effect of TLR4 signaling in thymoma on differentiation of $CD4^+$ T cells, we cocultured $CD4^+$ T cells from the blood of normal subjects with Thy0517 cells in which TLR4 signaling was activated or inhibited and then examined the ratio of Th17 and Treg cells by flow cytometry (Figure 13A-B). Compared with the control group at 24 h (1.09±0.06) and 48 h (1.22±0.05), Th17 cells were significantly increased at 24 h (p=0.0005) (1.73±0.04) and 48 h (p=0.0001) (1.96±0.04) after LPS treatment, and significantly reduced at 24 h (p=0.025) (0.72±0.16) and 48 h (p=0.028) (0.86±0.09) after TAK treatment. Additionally, compared with the control group at 24 h (8.76±0.46) and 48 h (9.06±0.93), Treg cells were significantly decreased at 48 h (p=0.031) (6.89±0.18) after LPS treatment and significantly increased at 24 h (p=0.0395) (10.83±0.44) after TAK treatment (Figure 13C) (Tables XI, XII).

Protein Expression of Foxp3 and RORγT in CD4⁺ T Cells Cocultured with Thy0517 Cells

We next examined changes in the expression of protein markers of Th17 and Treg cells (Figure 14A). RORγT was significantly increased at 24 h (1.94±0.18) (p=0.0006) and 48 h (1.76±0.23) (p=0.003) after stimulating TLR4 signaling by LPS and significantly reduced at 24 h (0.13±0.02) (p=0.010) and 48 h (0.40±0.10) (p=0.015) after suppressing TLR4 signaling by TAK. Foxp3 was reduced at 24 h and 48 h but only had significance at 24h (0.16±0.05) (p=0.048) in the LPS group. And Foxp3 had no significant results in the TAK group (Figure 14B) (Tables XIII, XIV).

Discussion

The thymus is an immune organ that provides a proper microenvironment to organize the development of thymocytes and instruct T cells to identify self from non-self, and eventually, exports

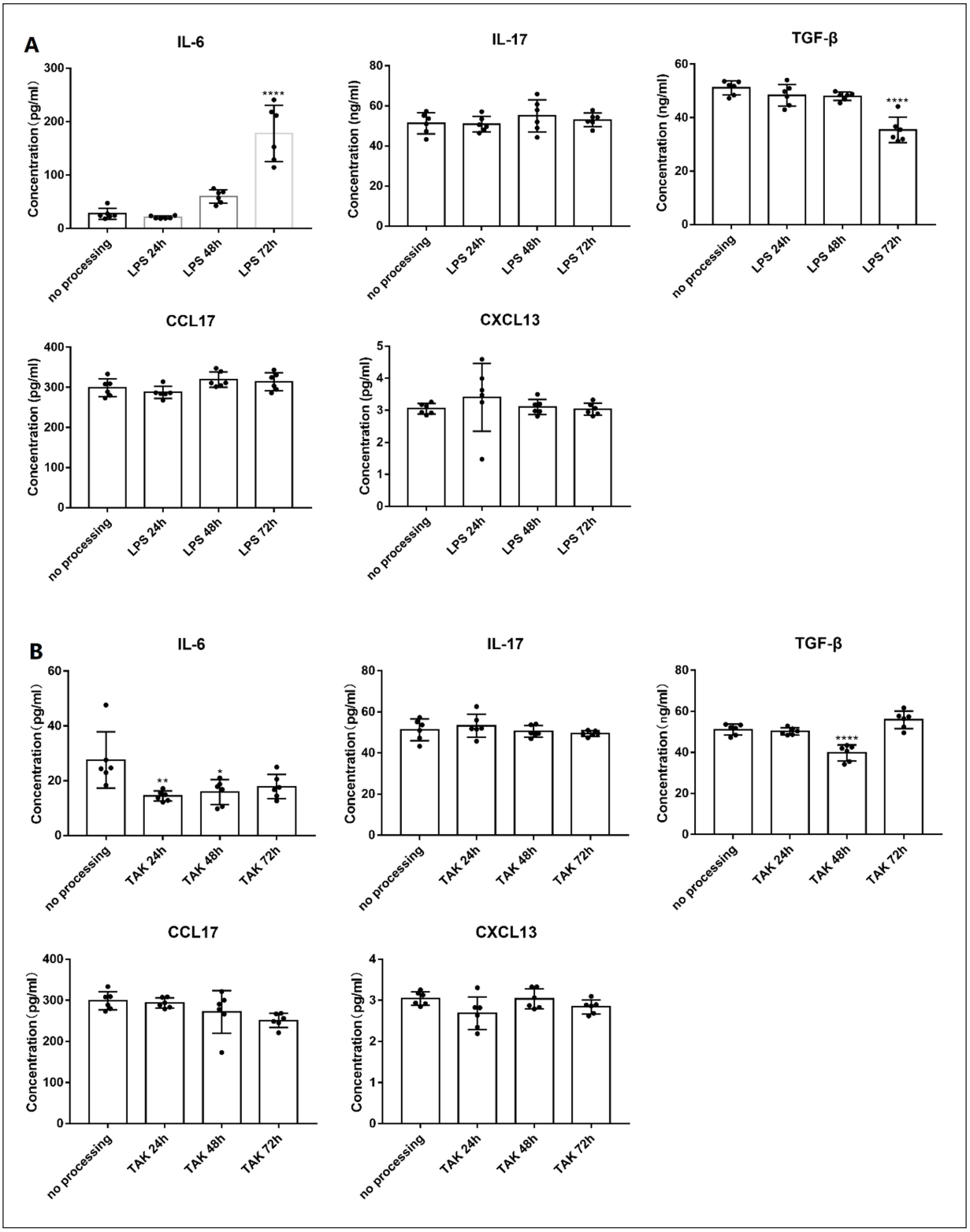


Figure 12. Expression of IL-6, TGF-β, IL-17, CCL17, and CXCL13 in Thy0517 cells after TLR4 regulation. **A**, Levels of IL-17, TGF-β, CCL17, CXCL13, and IL-6 in culture supernatants of Thy0517 cells after TLR4 stimulation. **B**, Levels of IL-17, TGF-β, CCL17, CXCL13, and IL-6 in culture supernatant of Thy0517 cells after TLR4 inhibition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table IX. Protein levels of IL-6, TGF-β, IL-17, CCL17, and CXCL13 in culture supernatants after TLR4 stimulation (one-way-ANOVA analysis).

Factors (ng/ml)	Control	LPS 24 h	LPS 48 h	LPS 72 h	*p*-value
TGF-β	51.12±2.65	48.39±4.11	47.99±1.56	35.39±4.79	**<0.001**
IL-6	27.59±10.27	20.84±2.59	59.80±12.38	177.86±52.45	**<0.001**
IL-17	51.29±5.24	50.91±3.85	54.99±8.01	53.06±3.38	0.556
CCL17	298.96±22.22	287.37±14.98	319.51±19.25	314.13±22.59	**0.045**
CXCL13	3.05±0.17	3.40±1.06	3.11±0.24	3.04±0.19	0.635

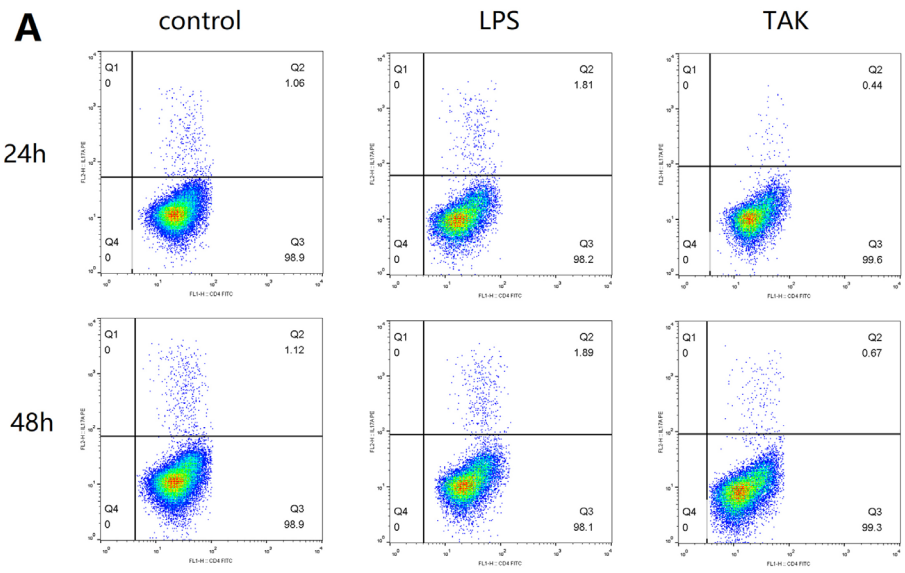
Table X. Protein levels of IL-6, TGF-β, IL-17, CCL17, and CXCL13 in culture supernatants after TLR4 inhibition (one-way-ANOVA analysis).

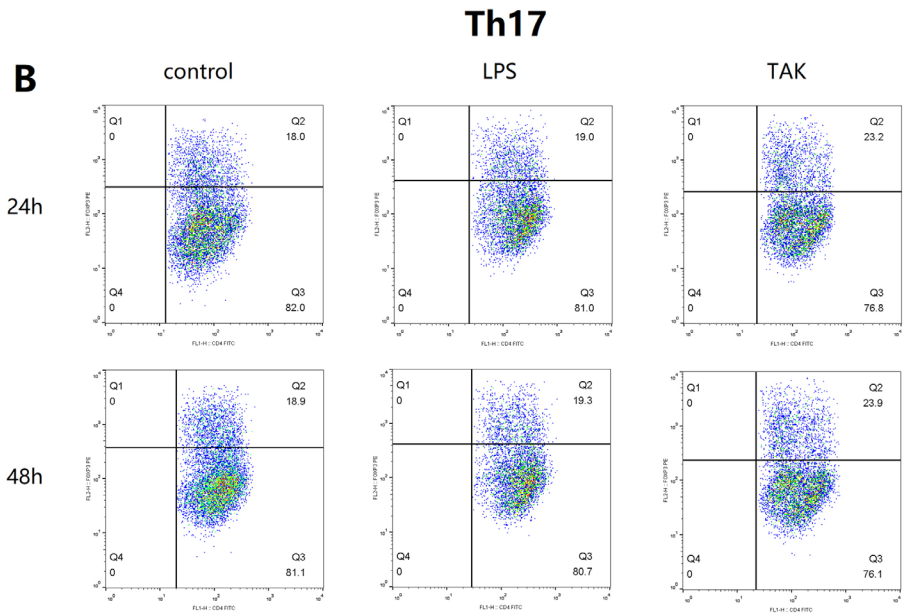
Factors (ng/ml)	Control	TAK 24 h	TAK 48 h	TAK 72 h	*p*
TGF-β	51.12±2.65	50.20±1.74	39.74±3.89	55.88±4.28	**<0.001**
IL-6	27.59±10.27	14.41±1.86	15.80±4.59	17.84±4.43	**0.006**
IL-17	51.29±5.24	53.30±5.63	50.56±2.80	49.52±1.32	0.463
CCL1	298.96±22.22	293.53±12.33	271.80±51.83	251.18±17.45	**0.047**
CXCL13	3.05±0.17	2.69±0.40	3.04±0.25	2.84±0.17	0.083

differentiated T cells to the periphery[20]. There is a balance between the necessity to remove pathogens and maintain immunological homeostasis to prevent autoimmunity. During T cell differentiation, any disturbance can break this balance and cause autoimmune diseases[21]. In recent years, attention has been focused on Treg cells and differentiation of pathological Th17 cells. A thymoma is a neoplasm in the thymus. Nearly 33% of thymoma patients also have autoimmune diseases, mainly MG[22,23], which indicates a strong link to the imbalance of Th17/Treg cells in accordance with our previous study[17]. Many studies[11,12,24,25] focusing on autoimmunity have suggested that TLR4 signaling is responsible for the imbalance of Th17/Treg cells and subsequent autoimmune diseases. Here, we investigated TLR4 and its downstream factors in the abnormal differentiation of Th17/Treg cells in MG thymoma.

The TLR4-MyD88-NF-κB pathway is highly active in many autoimmune diseases. TLR4 is an essential upstream sensor for NF-κB activation. It binds to MyD88 to activate NF-κB that is transferred to the nucleus to increase expression of proinflammatory cytokines TNF-α, IL-6, and IL-17[26]. The significant increases of TLR4, MyD88, and NF-κB in MG thymoma compared with simple thymoma suggested that the TLR4 signaling pathway plays an essential role in the pathogenesis of MG in thymoma patients. Among them, MyD88 showed a significant increase in protein level but no change in RNA level. This might be because the mRNA may have degraded when the protein reached its peak, or there are some other mechanisms we have not yet found. MyD88 levels in thymoma tissues are little studied, and further research is needed to be able to discover some deeper mechanisms of the TLR4-MyD88-NF-κB pathway in thymoma. Moreover, we did a co-localization experiment to see if these 3 proteins could be localized in one cell, and the results were not ideal, so we did not collect data and analyze statistics. The significant results of the IHC suggest errors in the experimental process or method.

There may be certain chemokines that participate in this signaling process. In our study, IL-6 and TGF-β were increased in MG thymoma tissue, indicating that IL-6 and TGF-β may also affect MG pathogenesis in thymoma patients. TGF-β is an important endogenous immune regulatory factor, controlling numerous cellular processes and exerting various effects such as immune suppression, anti-inflammation, and inhibition of tumor cell proliferation[27,28]. IL-6 is a typical cytokine that regulates numerous biological activities including the direction of naïve T cell differentiation[29]. IL-6 and TGF-β have cooperating effects. TGF-β promotes the differentiation of Th17 cells[30]m while IL-6 inhibits the generation of Treg cells[31] and accelerates Th17 cell development with the help of TGF-β[32]. Furthermore, IL-6 increases following NF-κB activation in the growth process of EAMG rats[33]. Therefore, IL-6 and TGF-β may function as a connection between the imbalance of Th17/Treg cells *via* TLR4 signaling in MG thymoma.





Treg

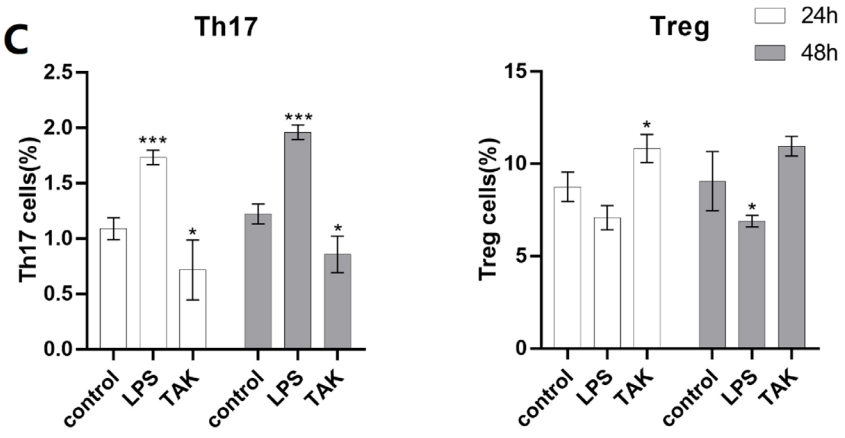


Figure 13. Differentiation of Treg and Th17 cells in Thy0517 cells after TLR4 regulation. **A**, Flow cytometric analysis of $CD4^+IL\text{-}17^+$ Th17 cells among $CD4^+$ T cells co-cultured with Thy0517 cells after TLR4 regulation. **B**, Flow cytometric analysis of $CD4^+CD25^{+-}$ $FoxP3^+$ Treg cells among $CD4^+$ T cells cocultured with Thy0517 cells after TLR4 regulation. **C**, Percentage of $CD4^+IL\text{-}17^+$ TH17 and $CD4^+CD25^{+-}$ $FoxP3^+$ Treg cells among $CD4^+$ T cells. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table XI. Percentage of Th17 and Treg cells in CD4+T cells after LPS treatment.

Target cells	Time	Control group	LPS group	*p*-value
Th17 cells	24 h	1.09±0.06	1.73±0.04	**0.0005**
	48 h	1.22±0.05	1.96±0.04	**0.0001**
Treg cells	24 h	8.76±0.46	7.08±0.38	0.1054
	48 h	9.06±0.93	6.89±0.18	**0.0311**

Table XII. Percentage of Th17 and Treg cells in CD4+T cells after TAK treatment.

Target cells	Time	Control group	TAK group	*p*-value
Th17 cells	24 h	1.09±0.06	0.72±0.16	**0.025**
	48 h	1.22±0.05	0.86±0.09	**0.028**
Treg cells	24 h	8.76±0.46	10.83±0.44	**0.0395**
	48 h	9.06±0.93	10.96±0.31	0.0619

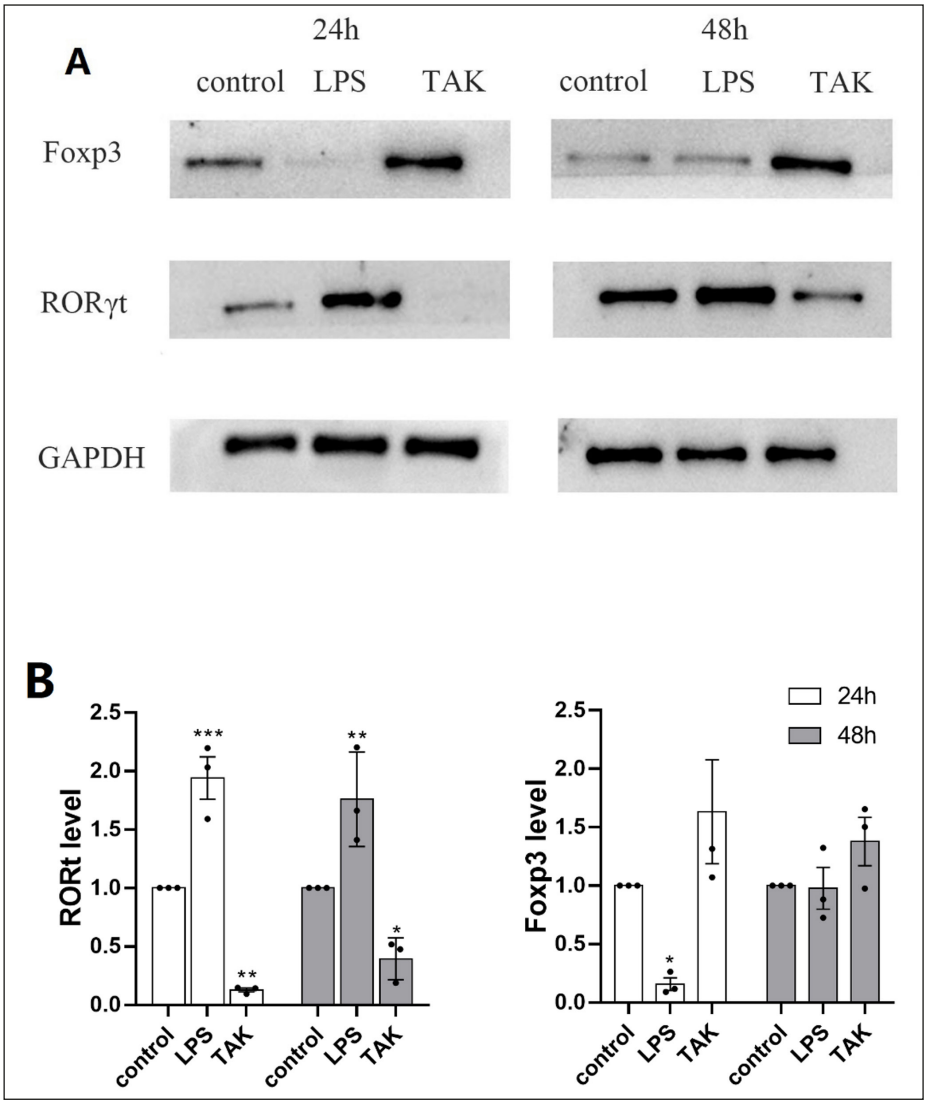


Figure 14. Expression of Foxp3 and RORγT in CD4⁺ T cells. **A**, Western blot of Foxp3 and RORγT in CD4⁺ T cells cocultured with Thy0517 cells. **B**, Expression levels of Foxp3 representing Treg cells and RORγT representing Th17 cells among CD4⁺ T cells cocultured with Thy0517 cells. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Table XIII. Protein relative levels of RORγT and Foxp3 after LPS treatment.

Proteins	Time	Control group	LPS group	*p*-value
RORγT	24 h	1	1.940±0.181	**0.0006**
	48 h	1	1.759±0.234	**0.0033**
Foxp3	24 h	1	0.157±0.053	**0.049**
	48 h	1	0.977±0.179	0.998

Table XIV. Protein relative levels of RORγT and Foxp3 after TAK treatment.

Proteins	Time	Control group	TAK group	*p*-value
RORγT	24 h	1	0.128±0.016	**0.0011**
	48 h	1	0.395±0.103	**0.015**
Foxp3	24 h	1	1.633±0.445	0.166
	48 h	1	1.378±0.206	0.553

IL-17, CCL17, and CXCL13 are related to MG thymoma. Increased IL-17 expression has been found in many autoimmune diseases, especially MG without exception[12,34,35]. In close association with IL-6, IL-17 also plays a role in development of Th17 cells, that in return produce IL-17 to function in their cell activities[3,36]. CCL17 expressed on functionally distinct subsets of T cells, such as Th17 and Treg cells[37], participates in the occurrence of multiple autoimmune diseases, including multiple sclerosis[38] and experimental autoimmune encephalomyelitis[39]. Abnormal recruitment of immune cells is directed to the MG thymus by CCL17 upon TLR4 overexpression[19]. CXCL13 is highly expressed in the MG thymus, but decreases after thymectomy[40], and its expression is higher in thymoma patients with MG than those without MG[41]. Therefore, we examined IL-17, CCL17, and CXCL13 in thymoma tissue and serum of MG thymoma patients compared with simple thymoma. We found that IL-17, CCL17, and CXCL13 were increased in MG thymoma tissues, demonstrating their relationship with MG thymoma. Our analysis indicates that the protein level of CXCL13 in thymoma tissue is not significant. Firstly, like what we have explained above about MyD88, there are many possible factors between mRNA and protein, so they are not exactly the same. Secondly, there is an increasing trend in the CXCL13 protein level in the MG group. However, due to the large individual differences and standard deviation, the result is statistically insignificant. Furthermore, the results obtained from the serum of MG thymoma patients were unexpected. This might be because the serum level of those cytokines and chemokines are correlated with the severity of MG and subtypes of thymoma[42,43]. Some non-specific interfering substances in blood serum and the constantly changing health and nutrient conditions of the patients may affect the results. Therefore, to investigate the role of TLR4 signaling pathway alteration in thymoma and its effect on Th17/Treg cells as well as the factors involved, we conducted an *in vitro* experiment. We focused on the expression of IL-6, IL-17, TGF-β, CCL17, and CXCL13 in Thy0517 cells after regulating TLR4 signaling. The results showed a significant increase of IL-6 and CCL17 and a decrease of TGF-β after TLR4 stimulation and a significant decrease of IL-6, CCL17, and TGF-β after TLR4 inhibition. It is worth noting that TGF-β showed the opposite trend of what we expected, which is supported by a study indicating that TGF-β also promotes the differentiation of Treg cells and functions adversely to IL-6[44]. Because of this contradictory function, TGF-β may show a different result. The remaining factors showed no significant differences, which might be due to nutrient depletion, contact inhibition of cells, or other mechanisms.

Next, we investigated the differentiation of Th17 and Treg cells in a transwell system of $CD4^+$ T and Thy0517 cells. The flow cytometry results showed that the expected changes were found in the Th17 cell percentage after TLR4 stimulation or inhibition in the transwell system, but only a slight difference was observed in Treg cells. Foxp3 and RORγT proteins are chosen to indicate Th17 and Treg cells. Foxp3 is a transcription factor that is specifically expressed by Treg cells and

required for their immune-suppressive function[45]. RORγT is also a transcription factor that is indispensable for Th17 cell differentiation and IL-17 expression[46]. Upon analyzing Th17 and Treg cells through flow cytometry, we found a significant difference in RORγT expression, but not in Foxp3. Treg cells lose Foxp3 expression and pathogenically transdifferentiate into Th17 cells in autoimmunity[47,48], which may explain this phenomenon. Overall, our data suggested that TLR4 signaling in MG thymoma affected the imbalance of Th17 and Treg cells.

Conclusions

In conclusion, our data indicate that TLR4 might play a role in the pathogenesis of MG thymoma. TLR4 is stimulated in thymoma, causing elevation of IL-6 and TGF-β, and the alterations of these factors lead to an increase of Th17 cells and a decrease of Treg cells, namely an imbalance of Th17/Treg cells, disturbing the stabilization of immune system and ultimately resulting in MG. In terms of IL-17, TGF-β, CCL17, and CXCL13, our study did not indicate any association with this mechanism.

Ethics Approval

The Ethics Committee of Tianjin Medical University General Hospital approved this study with No. ZYY-IRB2018-KY-018.

Informed Consent

All patients were informed and consented to this experiment.

Authors' Contributions

Yuan Chen designed the experiments. Ruiyu Zhang wrote the manuscript. Xuesong Zhang, Ruiyu Zhang, Yuanguo Wang, and Chao Lu performed the experiments, Zirong Wang and Yu Shi analyzed the data and prepared the figures, Peng Zhang provided support. All authors reviewed the manuscript.

Conflict of Interest

None of the authors have any conflicts of interest.

Funding

National Natural Science Foundation of China Youth Program (81801603), Tianjin Natural Science Foundation of Youth Program (19JCQNJC12000), and Beijing-Tianjin-Hebei Special Project (19JCZDJC64400).

Acknowledgments

We thank Kai Xiong, Yuxin Liu, and Zeyang Zhang (Department of Cardiothoracic Surgery, Tianjin Medical University General Hospital) for providing advice and assistance with experiments. We also thank Mitchell Arico from Liwen Bianji (Edanz) (https://www.liwenbianji.cn) for editing the language of a draft of this manuscript.

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