Imbalance of Th17 and Tregs in thymoma may be a pathological mechanism of myasthenia gravis

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Abstract

An imbalance in Th17 cells and Tregs may be an important cause of the pathogenesis of thymoma with myasthenia gravis (MG). In this study, 30 patients with simple thymoma and 30 patients with thymoma with MG were analyzed. Flow cytometry analysis of Th17 and Tregs in peripheral blood revealed that the percentages of Th17 in thymoma were lower than those in thymoma with MG, while the percentages of Tregs were higher than those in simple thymoma. Serum cytokine ELISA assays showed that IL-6 levels in simple thymoma were lower than those in MG patients. Further, Th17 and Tregs levels were detected by immunohistochemical double staining of thymoma tissue; the number of positive Th17 cells in thymoma with MG was higher than that in simple thymoma, while positive Tregs showed the opposite results. RORγt protein and mRNA expression in thymoma with MG were both higher than those in simple thymoma. FOXP3 protein and mRNA expression in the thymoma with MG group were lower than those in simple thymoma. The results of coculture of thymoma cells and CD4+ T cells showed that thymoma cells could promote the differentiation of Th17 cells and inhibit the Tregs. Overall, Th17 cells and related transcription factors and cytokines in thymoma with MG patients were higher than those in thymoma patients, whereas, Tregs showed the opposite results, the mechanism may be that thymoma can secrete IL6 and IL21. These findings indicated that imbalances in Th17/Tregs and RORγt/FOXP3 may account for the pathogeny of thymoma with MG.

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Short title: Imbalance of Th17 and Tregs in thymoma with MG

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Key Words:thymoma, myasthenia gravis, Th17, Treg, IL-6

Abbreviations used in this article: MG, myasthenia gravis; Th17, T helper type 17, Treg, T regulatory cells; RORγt, Retinoid-related orphan receptors gamma t; FOXP3, Forkhead transcription factor 3; PBMC, Peripheral blood monocyte; FCM, flow cytometer cytometry; ELISA, enzyme-linked immunosorbent assay.

Summary

An imbalance in Th17 cells and Tregs may be an important cause of the pathogenesis of thymoma with myasthenia gravis (MG). In this study, 30 patients with simple thymoma and 30 patients with thymoma with MG were analyzed. Flow cytometry analysis of Th17 and Tregs in peripheral blood revealed that the percentages of Th17 in thymoma were lower than those in thymoma with MG, while the percentages of

Tregs were higher than those in simple thymoma. Serum cytokine ELISA assays showed that IL-6 levels in simple thymoma were lower than those in MG patients. Further, Th17 and Tregs levels were detected by immunohistochemical double staining of thymoma tissue; the number of positive Th17 cells in thymoma with MG was higher than that in simple thymoma, while positive Tregs showed the opposite results. RORγt protein and mRNA expression in thymoma with MG were both higher than those in simple thymoma. FOXP3 protein and mRNA expression in the thymoma with MG group were lower than those in simple thymoma. The results of coculture of thymoma cells and CD4+ T cells showed that thymoma cells could promote the differentiation of Th17 cells and inhibit the Tregs. Overall, Th17 cells and related transcription factors and cytokines in thymoma with MG patients were higher than those in thymoma patients, whereas, Tregs showed the opposite results, the mechanism may be that thymoma can secrete IL6 and IL21. These findings indicated that imbalances in Th17/Tregs and RORγt/FOXP3 may account for the pathogeny of thymoma with MG.

Introduction

In the thymus, T-cell precursors differentiate to T regulatory cells (Tregs) and naïve T cells. Naive CD4+T cells differentiate into one of several CD4+T cell subpopulations including Th1, Th2, Th17, and T regulatory cells (iTregs). Over the past decade, T helper type 17 (Th17) cells producing interleukin-17 (IL-17) have emerged as the major pathogenic T cell subset in many pathological conditions[1,2]. In addition, the role of CD4+CD25+ Tregs in controlling the activity of Th17 and other T cell subsets has increasingly been realized [3]. Th17 and Treg development is governed by lineage-specific transcription factors. Retinoid-related orphan receptors gamma t (RORγt) is the master transcription factor of Th17 cell differentiation from naïve T cells and can induce the expression of IL-17A and IL-17F [4]. Forkhead transcription factor 3 (FOXP3) is a core subset-specific transcription factor expressed by Tregs, and its sustained expression is critical for the development and suppressive function of Tregs [5]. The reciprocal inhibition of RORγt and FOXP3 maintains the balance of differentiation to Th17 and Treg [6,7]. This balance plays a crucial role in the maintenance of immune homeostasis.

Th17/Treg imbalance has an important function in the pathogenesis of many autoimmune and inflammatory diseases, such as autoimmune arthritis, multiple sclerosis, intestinal inflammation, and sarcoidosis [8-11]. Similarly, myasthenia gravis (MG) is an organ-specific autoimmune disease characterized by muscle weakness, which is caused by autoantibodies directed against neuromuscular junctions. Th17/Treg imbalance is also an important factor in the pathogenesis of MG [12]. Higher levels of IL-17 were found in the serum of generalized AChR-MG patients than in control serum [13], and Treg functional defects associated with reduced expression of FOXP3 appeared to be more pronounced in MG patients [14].

Thymoma, which is a neoplasm of thymic epithelial cells, can cause defects in immune regulation and immune tolerance, resulting in autoimmune disease. The most common autoimmune disease associated with thymoma is MG. Wang et al. reported that an increase in Th17 cells and Th17-associated cytokines was observed in peripheral blood mononuclear cells (PBMCs) from MG patients with thymoma [15]. In our previous research, lower levels of FOXP3 mRNA and protein in thymoma tissue and CD4+CD25+FOXP3+ Tregs in PBMCs were associated with thymoma-related MG [16]. However, direct proof that thymoma induces a Th17/Treg imbalance is lacking. In this study, the imbalance of Th17/Treg was studied through an analysis of the relationship between Th17/Treg and transcription factors and cytokines in thymoma and peripheral blood. This study indicates the potential pathogenesis of thymoma-associated MG.

Materials and methods

Patients and samples

A total 60 patients with thymoma were recruited at the inpatient service of Tianjin Medical University General Hospital (Tianjin, China) between 2015 and 2017. All patients had undergone surgery but had not received chemotherapy or radiotherapy. Thirty patients had simple thymoma without other relative autoimmune diseases (Tm group), while 30 others were diagnosed with thymoma and AChR-MG (MG group). The ethics committee of Tianjin Medical University General Hospital approved this study.

Fasting venous blood samples were collected from individual patients before surgery and immunotherapy. Anticoagulated whole blood (3 mL) was examined by flow cytometry (FCM) within 4 h. Plasma samples were stored at -80 °C for enzyme-linked immunosorbent assay (ELISA). Thymoma tissue samples were collected from individual patients during surgery; one portion was fixed in 10% neutralized formalin overnight and was paraffin-embedded. The remaining tissues were frozen in liquid nitrogen.

Flow cytometry analysis of Th17 and Tregs in peripheral blood

PBMCs were processed by Ficoll density gradient centrifugation according to the manufacturer's instructions (Hao Yang Biological Manufacture Co., Ltd., Tianjin, P.R.Chian). PBMCs 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 analysis, cells were stained with APC-conjugated anti-human CD4 and PE-conjugated anti-human IL-17A antibodies (Miltenyi Biotec Technology & Trading (Shanghai) Co., Ltd. Shanghai, P.R.China). For Treg analysis, the cells were stained with FITC-conjugated anti-human CD4, APC-conjugated anti-human CD25, and PE-conjugated anti-human FOXP3 antibodies (Miltenyi Biotec). Isotype controls were used to correct compensation and confirm antibody specificity. Stained cells were analyzed by FCM (FACSCanto II; BD Biosciences, San Jose, CA, USA). Data analysis was performed using Cell Quest software (BD Biosciences).

Serum cytokines ELISA assay

The serum levels of IL-6, IL-21, IL-23, and TGF-β were quantified using commercially available ELISA kits according to the manufacturer's instructions (Proteintach Group Inc, Chicago, USA).

Immunohistochemical double staining analysis of Th17 and Tregs in thymoma

A two-color immunohistochemical double stain with CD4 and IL-17A primary antibodies (Th17 cells) and CD4 and FOXP3 primary antibodies (Tregs) was performed according to the manufacturer's protocol (KIT-9999: Fuzhou Maixin Biotechnology Development Co., Ltd. China). Paraffin-embedded thymoma tissue sections (4 μm) were de-waxed in xylene, dehydrated in ethanol, and subjected to antigen retrieval with Tris-EDTA under high temperature and high pressure for 3 min. Endogenous peroxidase activity was blocked by incubation with 3% $H_2O_2$for 10 min. Non-specific reactions were blocked by incubation with serum-free medium for 10 min at room temperature. Two sections from the same sample were incubated with anti-IL-17A antibody (1:100 dilution; ab136668, Abcam, Cambridge, UK) or anti-FOXP3 antibody (1:200 dilution; ab54501, Abcam) overnight at 4 °C. The following morning, the sections were incubated with goat anti-rabbit secondary antibody, and then were stained with BCIP/NBT (mandarin blue indicated positive staining). The slides were rinsed in PBS and incubated with double staining intensifier and serum-free medium for 10 min. The slides were then incubated with the second primary antibody, anti-CD4 (1:100 dilution; ab846, Abcam), overnight at 4 °C. Then, the sections were incubated with goat anti-rabbit secondary antibody and stained with AEC (3-amino-9-ethylcarbazole chromogen) solution and hematoxylin. Thus, the two primary antibodies could be easily differentiated by the various chromogens (IL-17A, mandarin blue immunolabeling in cytoplasm; FOXP3, mandarin blue immunolabeling in cytoplasm or nuclei; CD4, red immunolabeling in cell membrane), as well as by cellular stain localization. Double-positive cells were counted on five adjacent high-power fields (×400) of each section.

Analysis of RORγt and FOXP3 in thymoma

The immunohistochemical procedure of RORγt and FOXP3 staining as described above was used for staining of thymoma, with the exception of the second primary antibody. Sections were incubated with anti-human RORγt antibody (1:40 dilution; ab219496, Abcam) and anti-human FOXP3 antibody (1:50 dilution; ab54501, Abcam). The sections were then incubated with goat anti-rabbit secondary antibody and stained with DAB and hematoxylin. Brown granules in the nuclei were considered to represent a positive signal. The frequency of positive cells in five high-power fields (magnification, ×400, Leica DMIL, Germany) of each section was determined. More than 10% positive cells in each high-power field were found to have positive expression.

Real-time quantitative PCR (RT-qPCR) analysis of RORC (RORγt) and FOXP3 mRNA

in thymoma

Total RNA of thymoma and PBMC was prepared with TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, CA, USA). cDNA was reverse transcribed from total RNA (2 μg). cDNA was amplified with SYBR Premix Ex Taq (Takara Biotechnology, Dalian, China). The sequences of the PCR primer pairs for *RORC* (RORγt), *FOXP3* , and *GAPDH*were designed by GeneRunner, as shown in Table 1 (Aoke Biological Technology LLC, Beijing, China).

The amplification was performed at 95 °C for 1 min for pre-denaturation; 40 cycles of 94 °C for 30 s for denaturation, 56 °C for 15 s for annealing, and 72 °C for 30 s for extension; followed by a final extension at 72 °C for 10 min. The relative expression levels of target genes were determined using the 2^{-Cq} method.

Thymoma cell line culture and cytokines *ELISA assay* in culture medium

The thymoma cell line Thy0517 provided by cardiothoracic surgery department of Tianjin Medical University General Hospital (Patent number: ZL 2014 1 0312866.6), established from type AB thymoma with myasthenia gravis patient. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/high glucose medium (HyClone, Utah, USA) with 10% heat-inactivated fetal bovine serum (FBS) (NQBB, Victoria, Australia), 100 U/mL penicillin, 100 ug/mL streptomycin (Sigma-Aldrich, Missouri, USA) and in 5% CO2 incubator (Thermo, California, USA) at 37°C, the doubling time was 37 hours [17].

Thy0517 cells were subcultured into three culture bottles at the same time, the initial cell density was 20%. 1ml of culture medium was token from the 1st to 5th day after subculture, and blank culture medium was the control as the 0th day. The contents of IL-1β, IL-4, IL-6, IL-12, IL-21, IL-23 and TGF-β were quantified using commercially available ELISA kits according to the manufacturer's instructions (Proteintach Group Inc, Chicago, USA).

CD4+ T lymphocyte isolation by magnetic activated cell sorting (MACS)

PBMCs were obtained from the healthy people according to the manufacturer's instructions (Hao Yang Biological Manufacture Co., Ltd., Tianjin, P.R.Chian). PBMCs were added with 160μl buffer and 40 μl MASC CD4 microbeads (130-045-101, Miltenyi biotech, Bergisch Gladbach, Germany) and incubated at 4 for 15 min. The separation was performed according to the manufacturer's instructions. Cells were passed through a MACS column (type LS), the non-adherent cells were separated from CD4+ T cells by negative MACS selection. At least 10^6 CD4+ T cells were collected for later use.

Coculture ofthy0517 cells and CD4+ T lymphocytes

The full grown thy0517 cells in the culture bottle were digested and made into cell suspension. Cocultures of thy0517 cells and CD4+ T lymphocytes were performed using a six-well Transwell system (0.4-mm pore size membrane; Corning, Cambridge, MA, USA). 1×10^5 CD4+ T lymphocytes were added to the top portion, while equal volume of thy0517 cells were added on the bottom. Different concentrations of PHA(phytohemagglutinin) were added in the transwell top portion according to groups. Lymphocytes were harvested after 3 days, Th17 and Tregs were analyzed using flow cytometry in each well. This experiment was divided into 4 groups: Group A: CD4+ T lymphocytes; Group B: CD4+ T lymphocytes and thy0517 cells coculture; Group C: CD4+ T lymphocytes and thy0517 cells coculture and 10 μ g / ml PHA were added; Group D: CD4+ T lymphocytes and thy0517 cells coculture and 20 μ g / ml PHA were added. Repeat the above experiment for three times.

Statistical analysis

The data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using Student's t-test for paired samples, single-factor analysis of variance (one-way ANOVA) for multi-group data, and chi-squared test for categorical data. P -values <0.05 were considered statistically significant. Data were processed using SPSS 19.0 (IBM, Chicago, IL, USA).

Results

Patient's characteristics

A total of 60 patients were enrolled in the study, including 30 patients with simple thymoma (Tm group) and 30 patients with thymoma and MG (MG group). There was no significant difference between the two groups regarding sex, mean age, and WHO histological classification of thymoma (Table 2).

Frequencies of Th17 and Tregs in peripheral blood from thymoma patients

In our experiment, Th17 cells were identified as CD4+IL17+ T cells, and Tregs were identified as CD4+CD25+FOXP3+ T cells. The number of Th17 and Tregs in peripheral blood was measured by flow cytometry. As shown in Figure 1, the percentage of Th17 in peripheral blood from the TM group (2.02±1.22%) was lower than that from the MG group (3.80±2.00%) (P =0.006). Conversely, the percentage of Tregs in peripheral blood from the TM group (5.90 ± 2.21%) was higher than that from the MG group (4.14±1.28 %) (P =0.015).

Serum levels of IL-6, IL-21, IL-23, and TGF-β

IL-6, IL-21, IL-23, and TGF-β levels were determined in plasma samples by ELISA. As shown in Figure 2, IL-6 levels in the TM group (20.32±7.40 pg/ml) were lower than in the MG group (101.10±20.3 %) (P =0.01). IL-21, IL-23, and TGF-β levels in the TM group were lower than in the MG group, but this difference was not statistically significant.

Th17 and Treg levels in thymomas

We defined the phenotype of Th17 cells as CD4+ and IL-17+ lymphocytes in thymoma tissue and the phenotype of Tregs as CD4+ and FOXP3+ lymphocytes in thymoma tissue. The number of positive Th17 cells in thymoma from the MG group was 9.77±3.86, which was higher than that from the Tm group (4.93±2.13, P <0.001). The number of positive Tregs in thymoma from the MG group was 6.77±2.94, which was lower than that from the Tm group (9.93±4.40, P =0.002) (Fig. 3).

RORγt and FOXP3 protein expression in thymoma

The proportion of positive staining of RORγt in thymoma from the MG group was 76.7% (23/30), which was higher than that from the Tm group (43.3%, P =0.017). The positive staining proportion of FOXP3 in thymoma from the MG group was 53.3% (16 /30), which was lower than that from the Tm group (86.7%,P =0.010). (Fig. 4).

RORC and FOXP3 mRNA expression in thymoma

The relative expression level of*RORC* mRNA in thymoma from the MG group was 2.44±1.59, which was higher than that from the Tm group (P <0.001). The relative expression level of *FOXP3* mRNA in thymoma from MG group was 0.66±0.37, which was lower than that from the Tm group (P <0.001). The relative ratio of*RORC* /*FOXP3* in the MG group (4.29±2.84) was higher than that from the Tm group (P <0.001). (Fig. 5).

Cytokines *ELISA assay* in Thy0517culture medium

The contents of IL-6, IL-21 and TGF-β were higher in the culture medium of ty0517 cells, and the contents of IL-6 and IL-21 increased with days, while TGF-β decreased gradually. The contents of IL-1β, IL-4 and IL-23 were lower, and the changes were not significant with days. IL-12 was not detected in cell culture medium (Fig. 6)

The effect of ty0517 cells on the differentiation of human CD4 + T lymphocytes

After three days of coculture of thy0517 cells and CD4+ T cells, frequencies of Th17 and Tregs were analyzed by flow cytometry. The results showed that thy0517 cells could increase the percentage of Th17 cells (0.66 ± 0.07 vs 1.14 ± 0.08), and inhibit Tregs (3.10 ± 0.22 vs 2.41 ± 0.39). With the increase of PHA, the Th17 cells increased, while the increase of Tregs was not significant (Table 3 and Figure 7).

Discussion

Immune tolerance is an important physiological mechanism by which the body reduces or eliminates an immune response to particular agents. The current view is that Th17 cells have a more important role in the pathogenesis of autoimmune diseases and in mediating chronic inflammation [18], while Tregs maintain immune tolerance by inhibiting the activation and proliferation of CD4 + T cells [19]. The thymus is the organ in which T lymphocytes develop and mature. Thymoma is one of the important causes of MG. Therefore, the study of T lymphocyte characteristics can reveal the possible pathogenesis mechanisms of thymoma-related MG. This has already been shown in many studies, and an increase in Th17 cells and IL-17 was observed in PBMCs from MG patients with thymomas or thymic hyperplasia [15,20,21]. Dysfunction of Tregs and loss of FOXP3 expression are important mechanisms leading to autoimmune MG and are related to the severity of MG [12,14]. There are also reports of decreased Tregs and FOXP3 expression in thymoma patients with MG [16,22]. However, recent reports have focused on Th17 and Treg changes in peripheral blood and have not studied the changes in thymoma.

In our study, we found similar changes in Th17 and Treg numbers in both thymoma tissues and peripheral blood. This first indicates that the changes in Th17 and Tregs not only exist outside the thymus but also within it. Abnormal Th17 and Treg cells in thymoma may affect the differentiation of these cells in peripheral blood. Another possible explanation is that abnormal Th17 and Treg cells in peripheral blood migrate back to the thymus. In addition, our study showed that Th17 were increased and Tregs decreased in both the thymoma and peripheral blood of MG patients. This may be one of the causes of MG in thymoma, and may be a target for the future evaluation and treatment of MG.

The expression of RORγt and FOXP3, which are transcription factors in Th17 and Tregs, was also studied. The changes in RORγt and FOXP3 expression were consistent with the changes in Th17 and Treg. That is to say, the expression of RORγt was increased and the expression of FOXP3 was decreased in thymoma-related MG patients. However, the mRNA and protein expression of RORγt and FOXP3 were not detected in peripheral blood, possibly because their levels in peripheral blood are too low to be detected by our method. Some studies have shown that FOXP3 can directly affect RORγt and inhibit the differentiation of Th17 cells by reducing the binding of RORγt to the *IL17A* promoter region, specifically blocking the transcription of *IL17A* and leading to a significant reduction in IL-17A secretion [6,7].

Th17 and Treg cells are closely related and are mutually inhibited during their differentiation. IL-6 plays a critical role in regulating the balance of these two cell type cells. It can induce the differentiation of Th17 by promoting sequential engagement of the IL-21 and IL-23 pathways [23]. TGF-β can induce the differentiation of T cells into Th17 or Treg cells, while the differentiation of Tregs induced by TGF-β can be inhibited in the presence of IL-6 [24]. However, a large number of Th17 cells are produced by the co-induction of IL-6 and TGF-β, and abnormal regulation or overproduction of IL-6 can lead to the occurrence of autoimmune diseases [24,25]. An *in vitro* study showed that the expression of IL-17 mediated by RORγt increased and FOXP3 was blocked completely after the increase of TGF-β and IL-6 in cell culture medium [26]. Souroujon et al. reported that IL-6 has a crucial role in controlling the imbalance of Th17/Tregs in EAMG rats, and that treatment of myasthenic rats with neutralizing anti-IL-6 antibodies shifted this equilibrium in favor of Tregs and led to suppression of EAMG [27].

IL-6, IL-21, IL-23, and TGF-β levels in peripheral blood were detected in the current study. We found that only IL-6 was elevated in patients with MG compared with patients with thymoma alone, and there was no significant difference among the other three. We also found that the thy0517 cells can secrete IL-6 and IL-21, consume TGF-β by detecting the content of cytokines in culture supernatant. Furthermore, in order to explain the effect of thymoma cells on the differentiation of Th17 and Tregs, we used the coculture of Thy0517 cells and healthy CD4+ T cells. The results showed that thymoma could promote the differentiation of Th17 and inhibit the differentiation of Tregs. These results suggested that the elevation of IL-6 was associated with myasthenia gravis, and at the same time, it may be an important reason for the increase in Th17 cells and the decrease in Tregs. The mechanism may be related to the ability of the thymoma to secrete IL6 and inhibit TGF-β.

In conclusion, our results suggest that an imbalance in Th17/Tregs and RORγt/FOXP3 and increased expression of IL-6 are possible mechanisms leading to MG in patients with thymoma. The development of therapeutic drugs regulating various molecular targets in the Th17/Treg balance axis may become a new direction for the treatment of thymoma-related MG.

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Conflict of Interest:

The authors have no financial conflicts of interest.

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Table 1. PCR primer pair sequences for human *RORC*,*FOXP3*, and *GAPDH*

Gene name	Primer sequence

RORγt	Sense: 5'-CTCAAAGCAGGAGCAATGGA-3' Antisense: 5'-AGTGGGAGAAGTCAAAGATGGA-3'	160bp
FoxP3	Sense: 5'-AAGGAAAGGAGGATGGACG-3' Antisense: 5'-GCAGCAAGACAGTGGA-3'	124bp
GAPDH	Sense: 5'-TGGAGTCTACTGGCGTCTTC-3' Antisense: 5'-TTCACACCCATCACAAACATG-3'	152bp

Table 2. Patient's characteristics

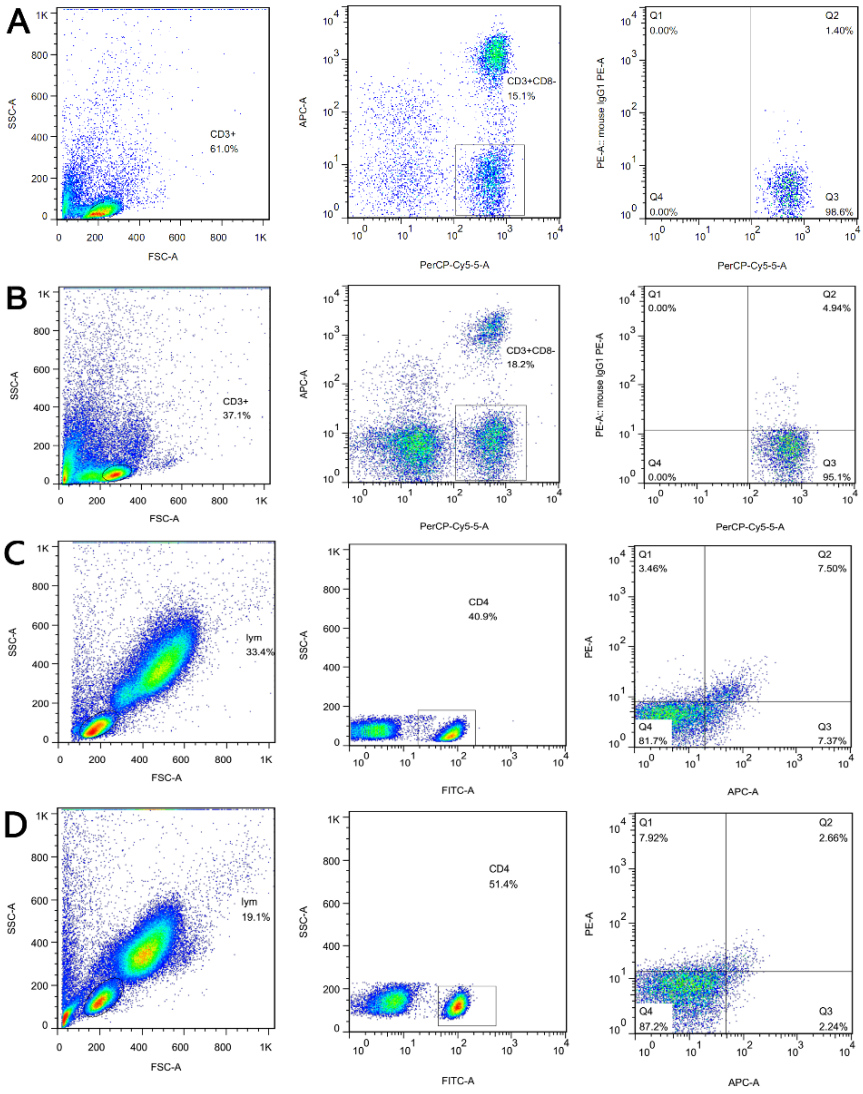
Characteristics	*MG Group*	*Tm Group*	X^2	*P*
	N=30	N=30		
Sex				
Male	14(46.7%)	17(56.7%)	0.60	0.44
Female	16(53.3%)	13(43.3%)		
Age	43.34(29-68)	49.69(28-64)	—	0.08
WHO histological classification				
A	5(16.7%)	6(20.0%)	0.67	0.96
AB	6(20.0%)	5(16.7%)		
B1	7(23.3%)	5(16.7%)		
B2	6(20.0%)	7(23.3%)		
B3	6(20.0%)	7(23.3%)		

Table 3 The percentage of Th17 and Tregs after coculture(%)

	Group A	*Group B*	*Group C*	*Group D*	*P* Value
CD3+CD4+T Cell	37.33±1.73	36.85±2.19	50.28±2.13	50.98±2.52	¡0.001
Th17	0.66±0.07*	1.14±0.08*	1.39±0.03*	1.72±0.08*	¡0.001
Tregs	3.10±0.22	2.41±0.39*	3.25±0.36	3.14±0.17	0.008

Group A: CD4+ T lymphocytes; Group B: CD4+ T lymphocytes and thy0517 cells coculture; Group C: CD4+ T lymphocytes and thy0517 cells coculture and 10 μ g / ml PHA were added; Group D: CD4+ T lymphocytes and thy0517 cells coculture and 20 μ g / ml PHA were added

*There was statistical significance between groups; The percentage difference of Th17 in each group was statistical significance; The percentage difference of Tregs between group A, C and D was no statistical significance.



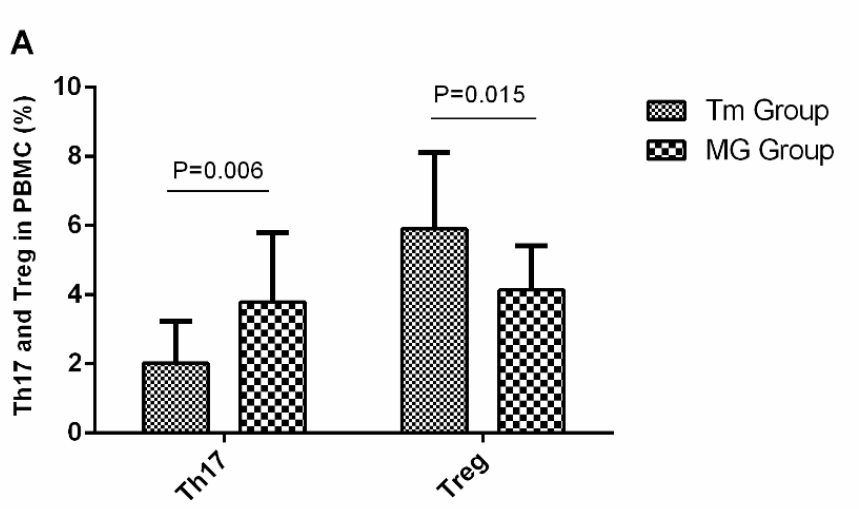
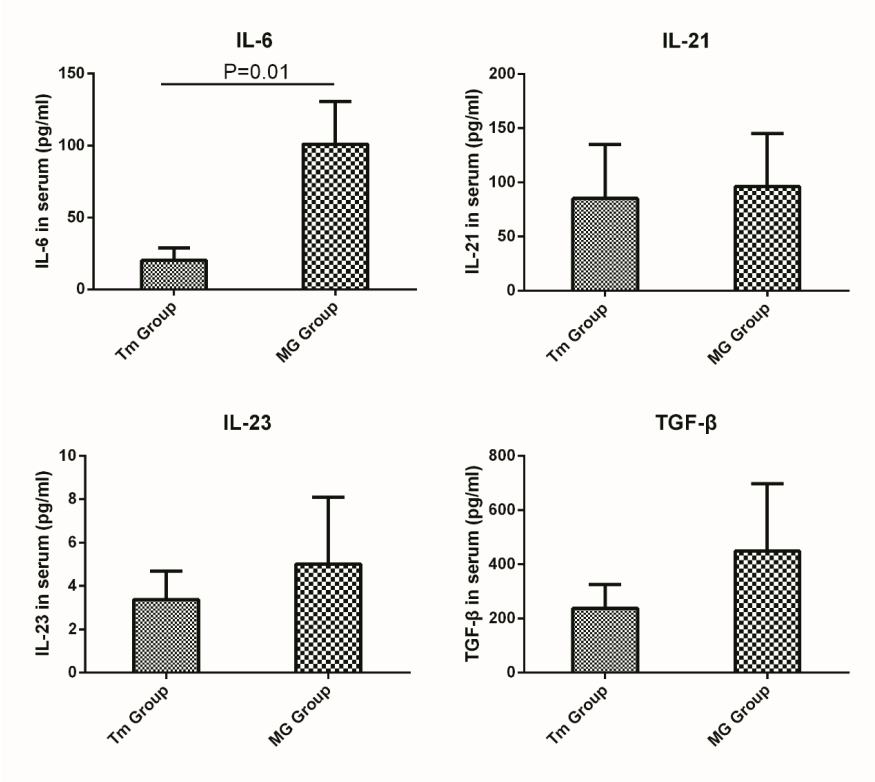


Figure 1. Flow cytometry analysis of Th17 and Tregs in peripheral blood. A–D Flow cytometry scatter plots of Th17 or Treg cells; the left scatter plots represent CD3+ lymphocytes in PBMCs, the middle scatter plots represent CD4+ T lymphocytes, and the Q2 quadrant in the right scatter plots represents Th17 or Treg cells. A, Th17 cells of Tm group; B, Th17 cells of MG group; C, Tregs of Tm group; D, Tregs of MG group; E, Percentage of Th17 and Treg cells in PBMCs.



Φιγυρε 2: Σερυμ λεελς οφ ΙΛ-6,ΙΛ-21, ΙΛ-23, ανδ ΤΓΦ-β ιν τηψμομα πατιεντς.

The serum levels of IL-6, IL-21, IL-23 and TGF-β were detected by ELISA assay. Only the IL-6 levels in Tm group were lower than those in MG group (P =0.01). Differences in IL-21, IL-23, and TGF-β levels between the two groups were not statistically significant.

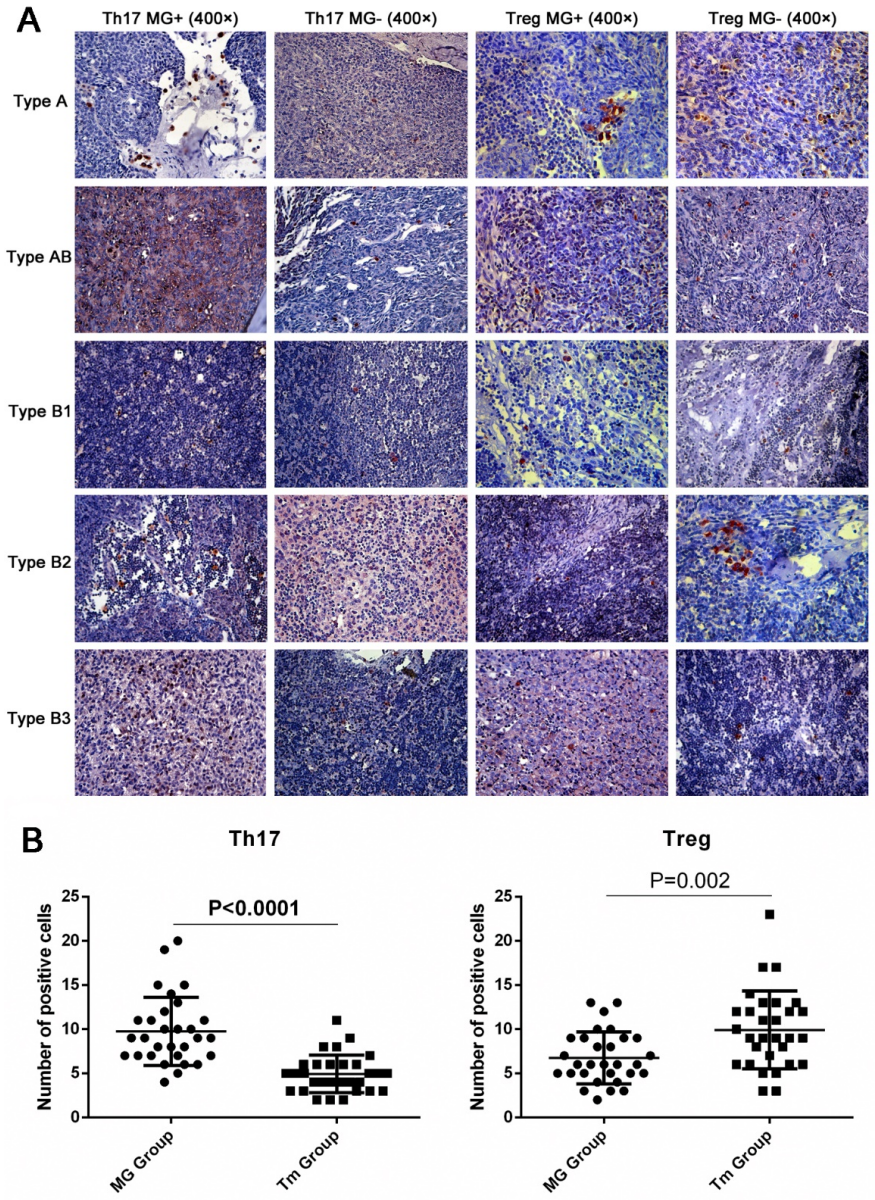


Figure 3. Th17 and Treg levels in thymomas.

A, Immunohistochemical double staining of Th17 and Tregs in each thymoma type. Positive cells are indicated in blue-purple in the nuclei and deep red in the periphery of cells, and were found in all types of thymoma; B, Number of Th17 and Tregs in thymoma. The number of positive Th17 cells in the MG group was higher than that in the Tm group. The number of positive Tregs in the MG group was lower than that in the Tm group.

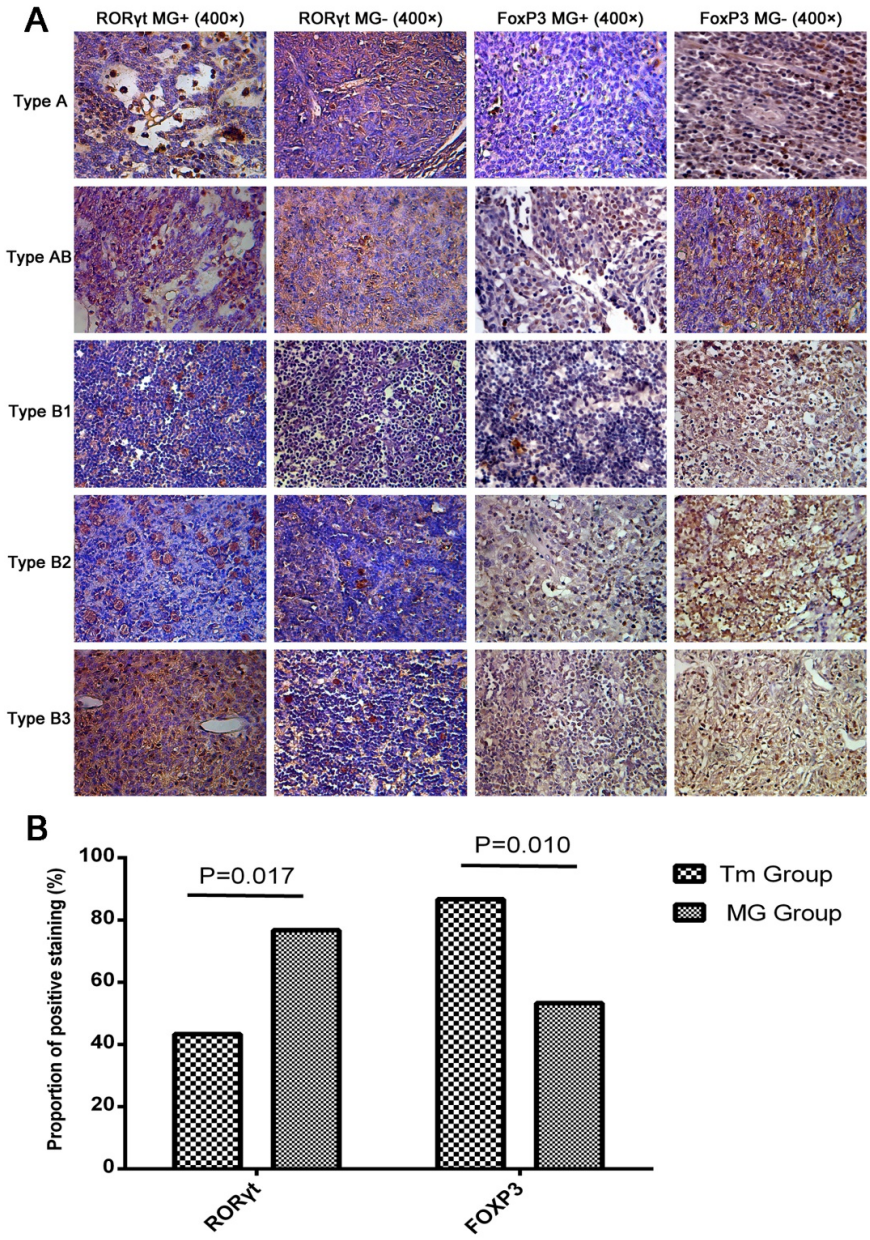


Figure 4: POPγτ ανδ ΦΟΞΠ3 προτειν εξπρεσσιον ιν τηψμομα.

A, Immunohistochemical staining of RORγt and FOXP3 in each thymoma type. Positive cells showed deeply stained brown nuclei. RORγt and FOXP3 protein expression was present in all types of thymoma, but not all thymoma cases.

B, The proportion of positive staining of RORγt and FOXP3 protein The proportion of positive staining of RORγt protein in thymoma was 76.7% and 43.3% in the MG group and Tm group, respectively. The positive staining proportion of FOXP3 protein in thymoma was 53.3% and 86.7% in the MG group and Tm group, respectively.

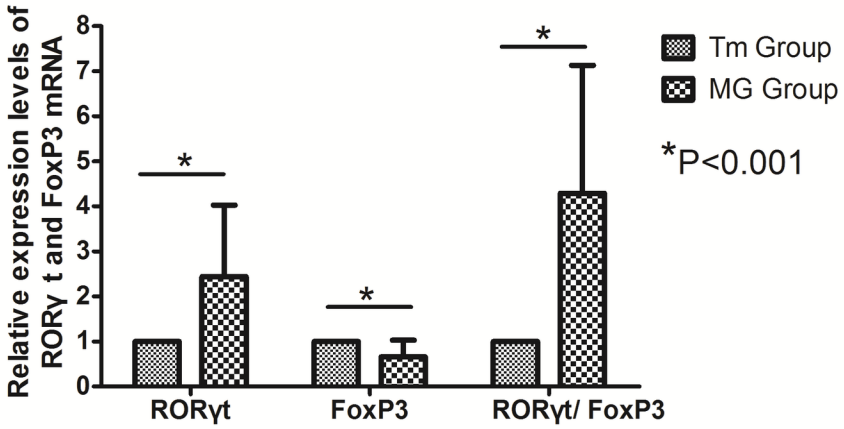


Figure 5: Relative expression levels of *RORC* and*FOXP3* mRNA in thymoma. *RORC* and *FOXP3* mRNA in thymoma were analyzed by RT-qPCR. The relative expression level of*RORC* mRNA in the MG group was higher than that in the Tm group, whereas *FOXP3* mRNA expression in the MG group was low. The difference in the *RORC* /*FOXP3* ratio between the two groups was significant.

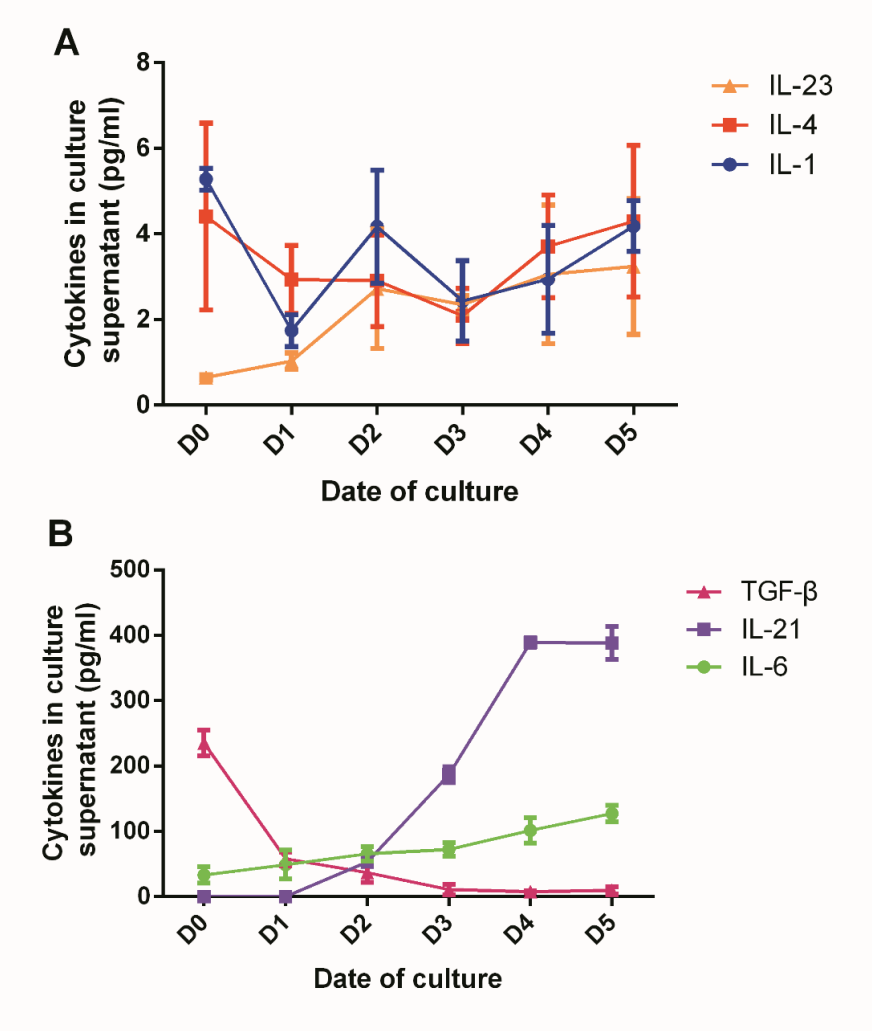


Figure 6 cytokine contents in Thy0517 culture medium

The contents of IL-1 β, IL-4, IL-6, IL-21, IL-23 and TGF - β in the supernatant of cell culture were analyzed from day 1 to day 5. The results showed that the contents of IL-1 β, IL-4 and IL-23 were lower, and the changes were not significant (A); the contents of IL-6, IL-21 and TGF - β were higher, and the contents of IL-6 and IL-21 increased gradually, while TGF - β decreased gradually (B).

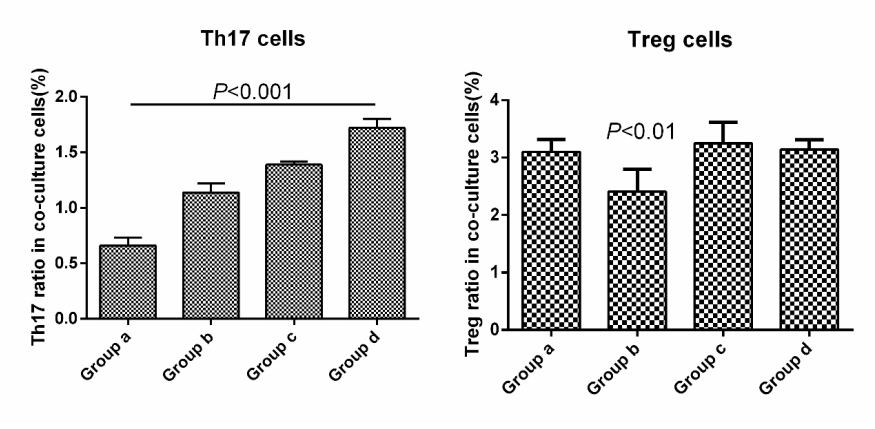


Figure 7 The percentage of Th17 and Tregs after coculture

Th17 cells: the percentage of Th17 cells in each group increased gradually, and there was statistical significance between each group; Treg cells: the percentage of Tregs in group B was the lowest, which was statistically significant compared with other groups. There was no statistical significance between group A, group C and Group D.