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RESEARCH ARTICLE

Decreased Expression of TIM-3 on Th17 Cells Associated with Ophthalmopathy in Patients with Graves' Disease

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Abstract: ***Purpose*:** Thyroid-associated Ophthalmopathy (TAO) is one of the most common orbital immunological diseases in adults. $CD4^+$ helper T (Th) cells play important roles in the pathogenesis of TAO. But the mechanisms regulating $CD4^+$ T cell activity is unclear. This study examines T cell immunoglobulin domain and mucin domain 3 (TIM-3) expression in helper T cell type 1 (Th1), Th17, and regulatory T cells in sufferers of TAO.

***Methods*:** Participants were divided into 3 groups: patients with TAO, patients with Graves' disease but without orbitopathy (GD), and healthy control patients (HC). Peripheral blood samples were collected for each patient in the designated group. Flow cytometry methods assessed the frequency of Th1 ($CD4^+IFN\text{-}\gamma^+$), Th17 ($CD4^+IL\text{-}17^+$), regulatory T cells ($CD4^+CD25^{hi}CD127^{lo}$), and TIM-3 protein expression. Mean fluorescence intensity (MFI) measured the magnitude of TIM-3 expression and the percentage of $TIM\text{-}3^+$ cells for each patient.

***Results*:** Compared to the GD group, TAO patients possessed higher frequencies of Th1 and Th17 cells in peripheral blood samples. The percentage of $TIM\text{-}3^+$ Th1 and Th17 cells was significantly lower in the TAO patients than the GD group. Across all patients sampled, $TIM\text{-}3^+$ cell percentage negatively correlated with Th1 cell frequency. Th1 and Th17 cells exhibited significantly decreased expression of TIM-3 in TAO patients compared to healthy controls. Regulatory T cells showed little TIM-3 expression and we observed no significant differences in frequency between groups.

***Conclusion*:** These results suggest a role for TIM-3 in the regulation of Th1 and Th17 cells and the pathogenesis of Graves' ophthalmopathy.

Keywords: T cell immunoglobulin domain and mucin domain 3, Thyroid-associated Ophthalmopathy, Graves' disease, $CD4^+$ helper T cells, autoimmune thyroid disease, immune tolerance.

1. INTRODUCTION

Thyroid-associated ophthalmopathy (TAO), also known as Graves' ophthalmopathy, represents one of the most common orbital diseases in adults. Epidemiological studies provide strong evidence for the association between TAO and Graves' disease (GD). About 90% of TAO patients are diagnosed with Graves' disease before or after the onset of ocular symptoms [1], while about 30-50% patients with Graves' disease develop orbitopathy [2, 3]. Unfortunately, the specific circumstances that cause Graves' disease patients to develop orbitopathy remain unclear and few risk factors are known.

TAO and GD belong to the class of autoimmune thyroid diseases and display a range of different manifestations across sufferers. Early research efforts identified helper T cell type 1 (Th1) as the predominant driver of inflammatory activities in TAO and GD pathogenesis [4-7]. Subsequently, more recent TAO and GD studies concluded that Th17 and regulatory T cells might also play a critical part [8-11]. Although changes in $CD4^+$ T cell subsets in TAO and GD were reported, the mechanism regulating $CD4^+$ T cell activity is unclear. Recent investigations suggest that T cell immunoglobulin domain and mucin domain 3 (TIM-3, also called Hepatitis A virus cellular receptor 2 or HAVCR2) is a central regulator in the maintenance of

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peripheral tolerance [12-14]. Initially discovered on the surface of murine Th1 cells [12], TIM-3 is upregulated on activated human $CD4^+$ T cells and limits immune activities by inhibiting proliferation and cytokine secretion. Blocking TIM-3 with antagonistic antibodies increases cytokine production by Th1 and Th17 cells [15]. Dysregulation of TIM-3 expression on $CD4^+$ T cells has been observed in patients with autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus [16, 17]. Expression of TIM-3 on regulatory T cells enhances their suppressive effect on pathogenic activities of Th1 and Th17 cells [18].

Based on TIM-3 activity in previous studies, we hypothesized that TIM-3 on $CD4^+$ T cells participates in the development of orbitopathy. To test this hypothesis, we examined TIM-3 expression on peripheral Th1, Th17, and regulatory T cells from patients with TAO, Graves' disease, healthy controls. In this study, we find evidence to suggest an association of TIM-3 with the development of TAO that may lead to novel treatment strategies.

2. MATERIALS AND METHODS

2.1. Participants and Sample Collection

Participants with TAO and healthy volunteers were recruited from Zhongshan Ophthalmic Center, while GD patients without orbitopathy were recruited from Guangdong General Hospital. Written consent form was obtained from the participants after complete explanation of possible consequences of the study. All patients with Graves' disease were undergoing or about to receive anti-thyroid treatment. TAO patients were diagnosed and assessed according to the established recommendations from European Group on Graves' Orbitopathy (EUGOGO) [19]. GD patients were diagnosed by standard clinical and laboratory testing by three qualified physicians. Trained technicians assessed thyroid function at the time of recruitment of all participants. Additional data collected at the time of recruitment included age, sex, and other vital statistics. The recruited healthy volunteers had no history or family history of autoimmune diseases and underwent no medical treatment. The study requirements excluded pregnant women and any patients with an infectious disease. Table **1** summarizes the demographic and clinical information of all participants.

2.2. Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from 2ml EDTA-k_2 treated blood sample with 1 × BD Pharm Lyse lysing solution (BD Pharmingen, USA) according to the instructions from the manufacturer. PBMCs were washed twice in 10ml phosphate buffered saline (PBS) (Corning, Manassas, VA, USA) with 1% fetal bovine serum (FBS) (Gibco, Life Technologies, USA) and collected by centrifugation at the speed of 300× g for 5 min.

2.3. Cell Culture

PBMCs were cultured at a density of 1×10^6/ml in RPMI-1640 medium (Gibco), supplemented with 10% FBS (Gibco), penicillin100U/ml, streptomycin100U/ml (both from Jinuo Co. Hangzhou, China), and stimulated with phorbol 12-myristate 13-acetate (PMA, 10ng/ml; Sigma, Saint Louis, MO, USA) and Ionomycin (1μM/ml; Sigma, Saint Louis, MO, USA) for 4 hours at 37°C in a 5% CO_2 incubator, with GolgiPlug (containing brefeldin A, 1μl/ml; BD Pharmingen, USA) in the last 2 hours. The cells were then harvested and washed with 2% FBS-PBS.

2.4. Cell Surface Antigen and Intracellular Cytokine Staining

Cultured PBMCs were incubated with fluorochrome-labeled monoclonal antibodies (mAbs) for 30 min at 4°C in the dark. Antibodies against human leukocyte surface markers CD4 and TIM-3 (eBioscience, San Diego, USA), CD25 and CD127 (BD Biosciences, USA) identified regulatory T cells. PMA and Ionomycin incubated PBMCs were collected and washed in 2% FBS-PBS, resuspended after centrifugation and distributed into 5ml polystyrene round-bottom tubes. Antibodies against surface marker CD4 and TIM-3

Table 1. Demographic and clinical information of patients and the healthy volunteers.

Clinical Parameter	HC	TAO	GD
Number of Subject	32	79	24
Age (yr) (mean± SEM)	40.22±2.96	43.10±1.36	34.17±2.47
Sex (F/M)	17/15	36/43	17/7
Duration of GD (months)	-	23.48±4.16	33.63±10.98
Duration of TAO (months)	-	15.37±2.26	-
Thyroid Function			
Euthyroid	32/32	46/79	4/24
Hyperthyroid	0/32	23/79	19/24
Hypothyroid	0/32	10/79	1/24

were first incubated with the cells followed by cell fixation and permeability with Cytofix Fixation Buffer and Perm/Wash Buffer (BD Biosciences, USA). Antibodies against interferon-γ (IFN-γ) and interleukin-17 (IL-17) (both from eBioscience, USA) were used to stain intracellular cytokines at 4°C for 30 min in the dark. The cells were then washed and resuspended in 2% FBS for flow cytometry analysis. Samples were examined with MACSQuant flow cytometer (Miltenyi, Germany).

2.5. Statistical Analysis

For normally distributed data, independent sample t-test or one-way ANOVA were conducted. Comparison involving non-normally distributed variables was conducted by Mann-Whitney U test or Kruskal-Wallis H test. The association between TIM-3 expression and cell frequencies was analyzed by Spearman rank correlation. Measurements were deemed significant based on a two-tailed hypothesis test with a significance level of 0.05.

3. RESULTS

3.1. TAO Patients Accumulate Greater Quantities of Th1 and Th17 Cells than GD Patients Without Orbitopathy

To identify the roles of Th1 and Th17 cells in the pathogenesis of TAO, we analyzed the frequency of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells in the peripheral blood from TAO patients, GD patients that didn't develop orbitopathy, and healthy volunteers (Fig. **1A**). The frequencies of Th1 and Th17 cells differed significantly among the three groups (Fig. **1B**). The frequency of Th1 and Th17 cells was significantly higher in the TAO group than in the GD group (For Th1, $P < 0.001$; Th17, $P < 0.05$). The frequency of regulatory T cells was similar in all groups ($P>0.05$). These data suggest that TAO and GD may present different underlying inflammatory mechanisms.

3.2. Th1 and Th17 Cells from TAO Patients Show Decreased Expression of TIM-3 Protein

We then aimed to investigate TIM-3 expression on these subsets. As shown in Fig. **2A** and **2B**, the proportion of $TIM\text{-}3^+$ Th1 and Th17 cells decreased significantly in TAO patients compared to GD patients (Th1, $P < 0.001$; Th17 $P < 0.05$). Regulatory T cells expressed extremely low levels of TIM-3, and showed no significant difference among three groups ($P > 0.05$, data not shown). The average level of TIM-3 expression on Th1, Th17, and regulatory T cells was measured by mean fluorescence intensity (MFI). As shown in Fig. **2B** and **2C**, the expression of TIM-3 on Th17 was significantly lower in TAO patients as compared to healthy controls ($P<0.05$). TIM-3 protein expression in Th1 cells showed no significant difference between TAO samples and healthy controls. In addition, Th1 cells from GD patients have higher amounts of TIM-3 expression than Th1 cells from TAO patients ($P<0.05$). No significant difference was observed in the expression of TIM-3 on regulatory T cells among the three different groups.

3.3. TIM-3 Expression Negatively Correlated with Th1 and Th17 Cell Frequency

To investigate the relationship between TIM-3 expression and the PBMC frequency of Th1 and Th17 cells, we performed Spearman rank correlation analysis. Our findings demonstrate that the $TIM\text{-}3^+$ proportion of Th1 and Th17 cells negatively correlates with the specific immune cell frequency in all participants (Fig. **3A** and **3B**; $r = -0.519$, $P <0.001$ for Th1; $r = -0.366$, $P < 0.001$ for Th17). Separate experimental measurement by MFI showed the same trend with the average level of TIM-3 expression negatively correlating with Th1 ($r = -0.364$, $P < 0.001$) and Th17 ($r =-0.270$, $P <0.01$) cell frequency (Fig. **4A** and **4B**). Although a similar trend was observed in regulatory T cells, TIM-3 expression level had no significant correlation with cell frequency (Fig. **4C**; $r = -0.079$, $P>0.05$). These data suggest that TIM-3 plays a role in mediating inflammation by regulating Th1 and Th17 cell frequency.

4. DISCUSSION

Thyroid-associated ophthalmopathy is a potentially sight-threatening autoimmune orbital disease in adults. Often happened to patients with Graves' disease, TAO is also called Graves' ophthalmopathy. TAO and Graves' disease represent two common and debilitating autoimmune thyroid diseases. Both physicians and epidemiologists have long considered the close relationship between these two diseases. Unraveling the pathogenesis of thyroid-associated ophthalmopathy remains a challenge despite extensive research efforts.

According to previous studies, Graves' disease is mediated by $CD4^+$ T cells, predominantly Th1 cells [20]. Ocular signs and symptoms of TAO are mainly caused by enlargement of orbital fat and extraocular muscles, as well as extensive hyaluronan synthesis in response to local infiltration of inflammatory cells [21]. With the discovery of large amounts of T cells in biopsies of extraocular muscles from TAO patients, Pappa *et al*. claimed that T cells were important initiators of orbital inflammation [22]. Coculture of autologous T cells with orbital fibroblasts from TAO patients *in vitro* suggests the stimulatory role of pathogenic T cells, especially $CD4^+$ T cells [7, 23]. Despite the shift of Th1/Th2 balance observed in orbital tissues by early studies [7, 24], similar alterations in cell frequency and cytokine levels were also observed in the peripheral blood [5, 8] suggesting parallel processes in local and systemic immunity.

A study by Xia *et al*. reported the higher percentage of Th1 cells in the peripheral blood of TAO patients than that of GD patients and healthy volunteers, suggesting that Th1 cells contribute to the development of ophthalmopathy [5]. Xia *et al*. also observed that the percentage of Th1 and Th2 cells was not influenced by

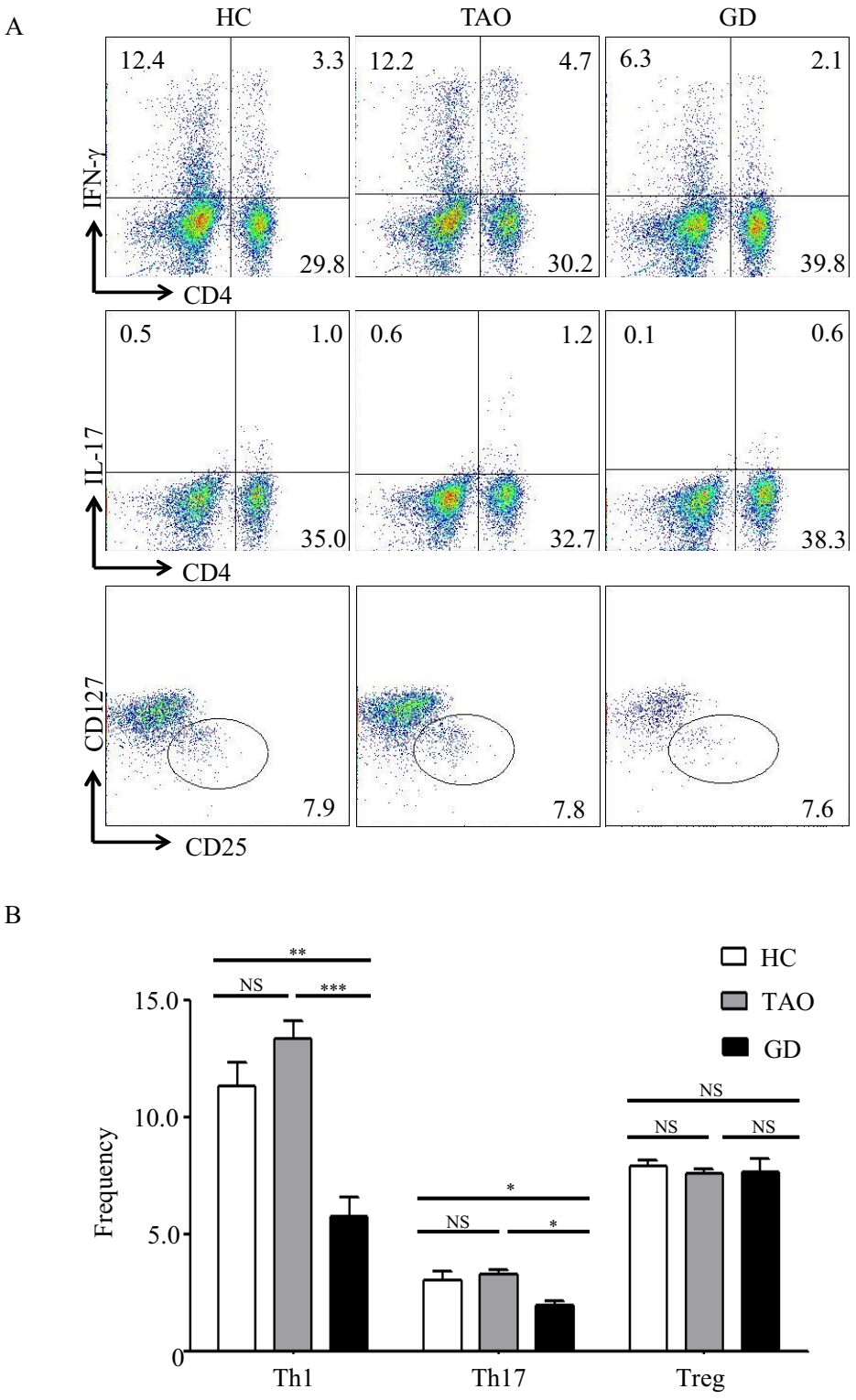

Fig. (1). Cell surface marker and intracellular cytokine staining.

(**A**) Lymphocytes were delineated based on side and forward scatter properties (not shown). $CD4^{+}IFN\text{-}\gamma^{+}$, $CD4^{+}IL\text{-}17^{+}$, and $CD4^{+}CD25^{hi}CD127^{lo}$ cells were identified as Th1, Th17, and regulatory T cells, respectively. (**B**) Summary of Th1, Th17 and regulatory T cell subset frequency. The frequency of Th1, Th17 and regulatory T cells in HC, TAO and GD group were shown as Mean ± SEM. Data were analyzed with multiple linear regressions and corrected for age and sex. SEM: Standard Error of Mean; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, NS: not significant.

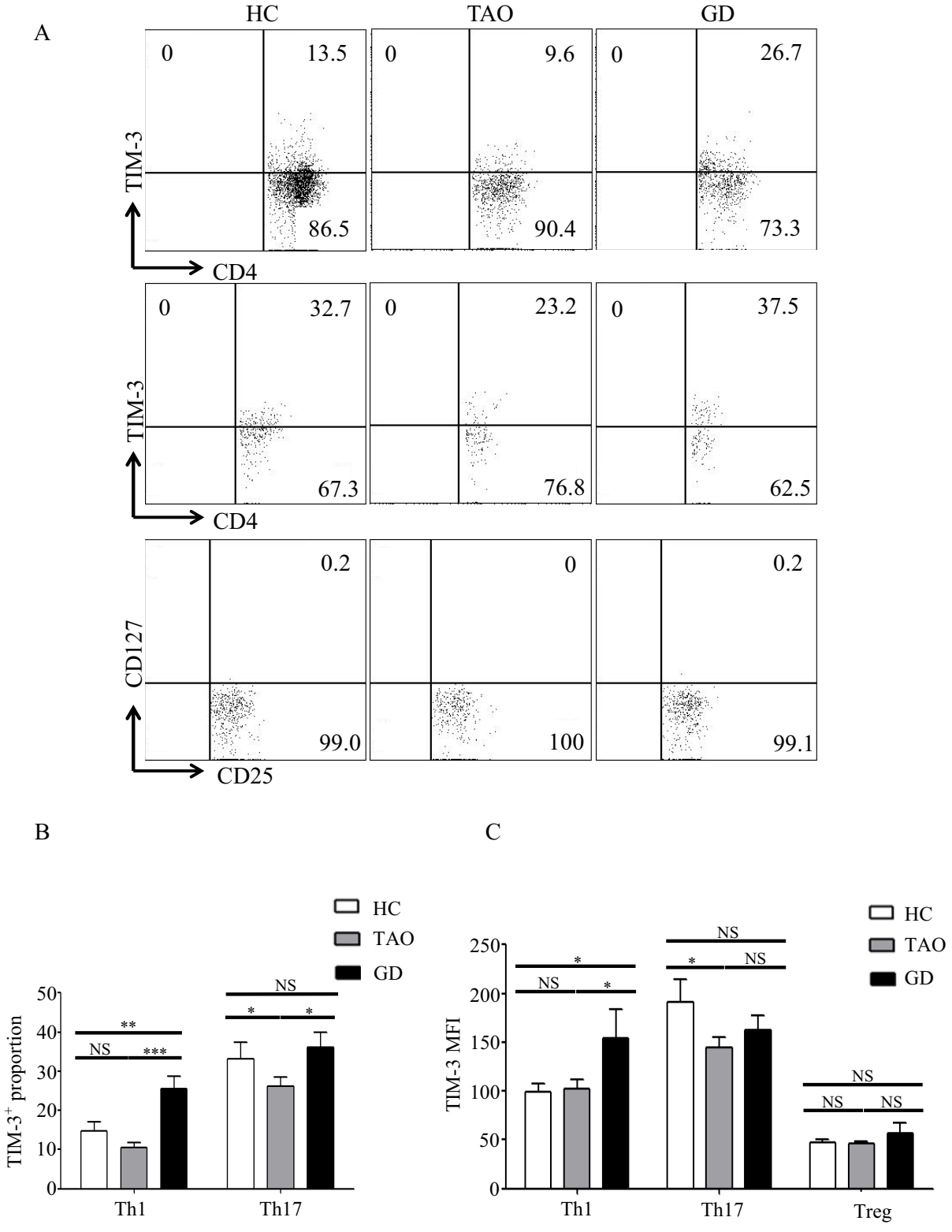

Fig. (2). TIM-3 expression on Th1, Th17 and regulatory T cells.
(**A**) Th1, Th17 and regulatory T cells were gated for TIM-3$^+$ proportion in HC, TAO and GD group. (**B**) Proportion of TIM-3$^+$ cells in Th1 and Th17 were summarized. (**C**) Average level of TIM-3 expression measured by MFI. Data analyzed and shown as Mean±SEM. Comparison was made with multiple linear regressions and corrected for age and sex. SEM: Standard Error of Mean; *P<0.05, **P<0.01, ***P<0.001, NS: not significant.

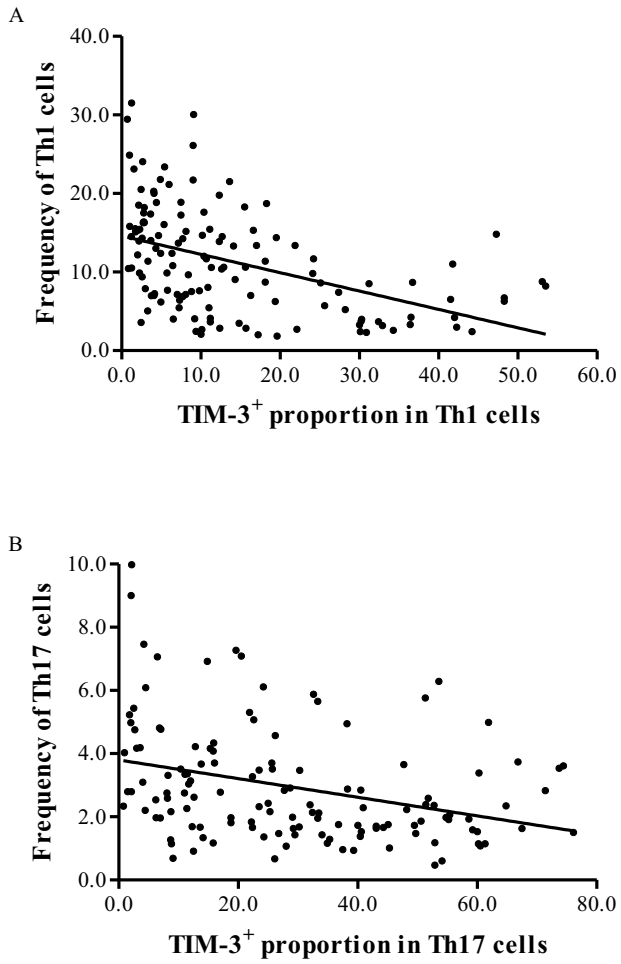

Fig. (3). Correlation between TIM-3$^+$ proportion and the frequency of Th1 and regulatory T cells.
The association between TIM-3$^+$ proportion and cell frequency was analyzed by Spearman rank correlation. There was a negative correlation between TIM-3$^+$ proportion and the frequency of (**A**) Th1 ($r = -0.519$, $P < 0.001$) and (**B**) Th17 cells ($r = -0.366$, $P < 0.001$) in all participants.

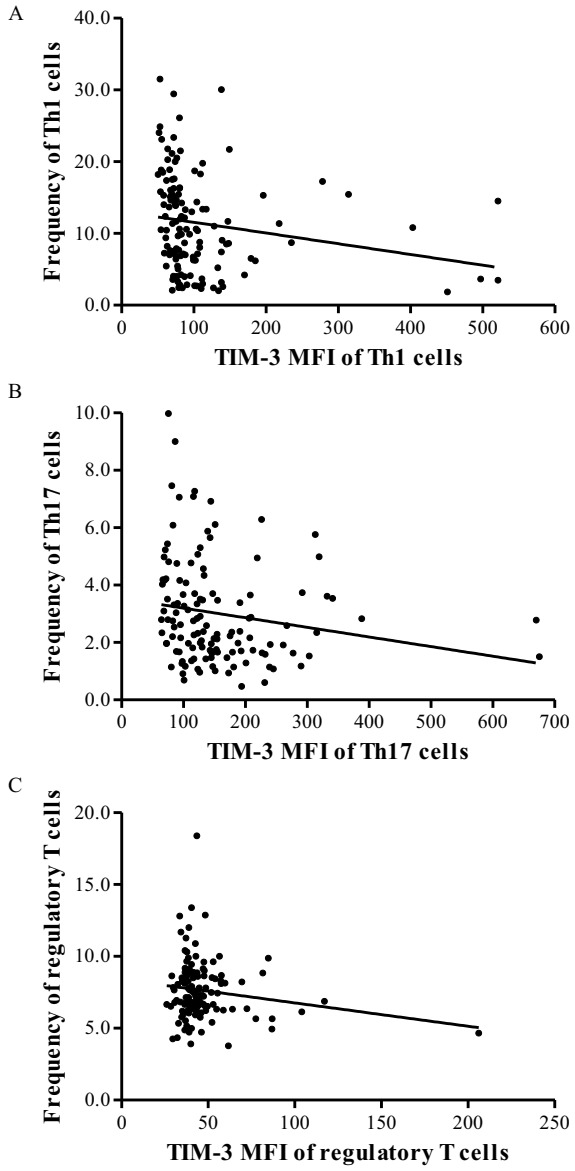

Fig. (4). Correlation between the average levels of TIM-3 expression and the frequency of each subset.
The association between TIM-3 expression and frequency of Th1, Th17, and regulatory T cells were analyzed by Spearman rank correlation. TIM-3 expression measured by MFI was negatively correlated with the frequency of (**A**) Th1 ($r = -0.364$, $P < 0.001$) and (**B**) Th17 cells ($r = -0.270$, $P < 0.01$). (**C**) TIM-3 expression on regulatory T cells showed no significant correlation with the frequency ($r = -0.079$, $P > 0.05$).

factors regarding thyroid function. The reason for the percentage change of Th1 cells was not further explored. In more recent studies, Th17 cells emerged as a potent inducer of autoimmune inflammation. More evidence showed Th17 participation in the development of orbital disease. It was demonstrated that orbital fibroblasts release chemokines and cytokines that promoted T cell migration to the orbit, leading to inflammation and orbitopathy [21]. Expression of IL-17A and IFN-γ was enhanced in orbital tissues in TAO patients [25]. IL-17A stimulated the expression of Regulated upon activation, and normal T-cell expressed and secreted (RANTES) by binding to orbital fibroblasts, which was much relevant to inflammatory disorders [25]. Besides, IFN-γ and IL-17A producing T cells promoted fibrosis in CD90$^+$ orbital fibroblasts, suggesting the pathogenic role of Th1 and Th17 cells [26]. Differences in Th1 and Th17 cell frequency as well as in cytokine levels in the peripheral blood have also been observed between patients with Graves' disease and TAO [8, 10, 11, 25], but the mechanisms driving these changes remain unclear.

Originally, TIM-3 was regarded as a uniquely expressed marker for Th1 cells [12]. By binding to its ligand, galectin-9 (LGALS9 in human), TIM-3 serves as a negative regulator in Th1 mediated immune responses [27]. Impaired expression or blockade of TIM-3 on these cells could lead to failure of peripheral

tolerance. In the murine model of experimental autoimmune encephalomyelitis, inflammatory activity and severity of disease increased after administration of a TIM-3 antibody [12]. Galectin-9 suppressed Th1 immunity *in vivo* and caused selective loss of IFN-γ producing cells. Hastings *et al*. later reported TIM-3 expression on Th17 cells [15]. Human $CD4^+$ T cells produce higher levels of Th1 and Th17 cytokines when stimulated with a TIM-3 antagonistic antibody and anti-CD3/anti-CD28 [15]. Change of TIM-3 expression on $CD4^+$ T cells was found to be associated with other human diseases such as multiple sclerosis and immune thrombocytopenia [16, 28]. Therefore, we hypothesized that TIM-3 might participate in the pathogenesis of autoimmune thyroid diseases.

In this study, we collected peripheral blood samples from TAO patients, GD patients without orbitopathy, and healthy volunteers. Frequency of Th1, Th17, regulatory T cells, and the expression of TIM-3 in PBMCs were measured by flow cytometry in each of the three groups. We found that TAO patients exhibited significantly higher frequencies of Th1 and Th17 cells and a significantly lower proportion of $TIM\text{-}3^+$ immune cells than GD patients without orbitopathy. Further analysis revealed a negative correlation between TIM-3 expression and helper T cell frequency indicating that reduced expression of TIM-3 in TAO patients may be associated with the susceptibility of orbitopathy in Graves' disease patients.

As an important negative regulator in T cell immunity, diminished TIM-3 expression in TAO patients may represent a defect in immunoregulation. In a recent study by Leskela *et al*., decreased expression of the TIM-3 ligand galectin-9 was observed on peripheral antigen-presenting dendritic cells (DC) from patients with Graves' disease, mainly in those with ophthalmopathy [29]. Our study found significantly lower TIM-3 expression on Th1 and Th17 cells in patients with ophthalmopathy compared to Graves' disease patients without orbitopathy. Reduced expression of TIM-3 and galectin-9 in TAO patients represented a weakened negative regulation mechanism. As has been observed in other autoimmune diseases [16, 30], it could result in the failure of peripheral tolerance, and enhanced inflammatory activities of Th1 and Th17 cells. It was also in accordance with previous findings regarding increased levels of Th1 and Th17 cytokines [8]. Paralleled with the peripheral changes, Fang *et al*. reported enhanced expression of IL-17A in orbital tissues in TAO patients, which in turn resulted in more T cell recruitment in the orbit and accelerated the orbitopathy by interacting with orbital fibroblasts[25]. These findings suggest that by influencing Th1 and Th17 frequencies, changes in TIM-3 expression are involved in the development of orbitopathy in patients with Graves' disease.

CONCLUSION

In conclusion, our findings revealed a reciprocal relationship between TIM-3 expression and the frequency of Th1 and Th17 cells in patients suffering from autoimmune thyroid diseases. Relatively higher levels of TIM-3 expression in GD patients may represent a protective factor that reduces the probability of an ophthalmopathy. These results motivate the need for further study of the effect of TIM-3 in the development of orbitopathy in Graves' disease and highlight the need for a successful animal model to further explore the immunoregulatory roles of TIM-3. More work is warranted to identify the molecular mechanisms underlying the regulation of TAO by TIM-3.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The design of this protocol follows the tenets of the Declaration of Helsinki, approved by the Institutional Review Boards of Zhongshan Ophthalmic Center, Sun Yat-sen University (No. 2016MEKY013).

HUMAN AND ANIMAL RIGHTS

All the procedures involving human subjects were compliant with the ethical guideline of the 1975 Declaration of Helsinki and its subsequent revisions. No animals were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

All participants provided informed consent for enrollment in the study after an explanation of possible consequences provided by a qualified physician.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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ABBREVIATIONS

TAO	=	Thyroid-associated Ophthalmopathy
TIM-3	=	T cell immunoglobulin domain and mucin domain 3
HAVCR2	=	Hepatitis A virus cellular receptor 2
GD	=	Graves' disease
HC	=	Healthy control
Th1	=	Helper T cell type 1
IFN-γ	=	Interferon-γ
IL-17	=	Interleukin-17
PBMCs	=	Peripheral blood mononuclear cells
MFI	=	Mean fluorescence intensity
DC	=	Dendritic cells

RANTES = Regulated upon activation, normal T-cell expressed and secreted

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