Implications of FoxP3-positive and -negative CD4+ CD25+ T cells in Graves' ophthalmopathy

Kazuhiko Matsuzawa1), Shoichiro Izawa2), Tsuyoshi Okura2), Shinya Fujii3), Kazuhisa Matsumoto2), Kyoko Shoji2), Risa Nakamura2), Keisuke Sumi2), Yohei Fujioka3), Akio Yoshida4), Chiaki Shigemasa5), Masahiko Kato2), Kazuhiro Yamamoto2) and Shin-ichi Taniguchi1)

1) Department of Regional Medicine, Tottori University Faculty of Medicine, Yonago 683-8504, Japan
2) Endocrinology and Metabolism, Department of Molecular Medicine and Therapeutics, Tottori University Faculty of Medicine, Yonago 683-8504, Japan
3) Division of Radiology, Department of Pathophysiological and Therapeutic Science, Faculty of Medicine, Tottori University, Yonago 683-8504, Japan
4) Division of Regenerative Medicine and Therapeutics, Tottori University Graduate School of Medicine, Yonago 683-8504, Japan
5) Internal Medicine, Tottori Municipal Hospital, Tottori 680-8502, Japan

Abstract. Graves' ophthalmopathy (GO) is a common manifestation of Graves' disease (GD); however, its pathogenesis is not well understood. Recently, the dysregulation of regulatory T cells (Tregs) has been thought to be closely associated with the pathogenesis and clinical symptoms of autoimmune disease. We therefore evaluated whether T cell subsets, including Tregs, are associated with GO pathogenesis and clinical symptoms. In this observational study we evaluated 35 GD patients with overt ophthalmopathy (GOs) and 28 patients without ophthalmopathy (non-GOs). Fifteen healthy euthyroid patients served as healthy controls (HCs). Peripheral blood mononuclear cells from GOs, non-GOs and HCs were analyzed for CD4, CD25, and FoxP3 expression using flow cytometry. We also evaluated their correlation with disease activity according to the clinical activity score (CAS) and magnetic resonance imaging (MRI) findings. Disease severity was evaluated using the NOSPECS score, and clinical progression of GO was followed for 24 weeks. The main outcome measures were the frequencies of FoxP3-positive and -negative CD4+ CD25+ T cells at study outset, namely Tregs and effector T cells (Teffs), respectively. GOs had higher frequencies of Teffs (30.8±8.4%) than non-GOs (19.4±7.1%) and HCs (22.7±7.9%). Notably, patients with improved GOs had lower frequencies of Tregs (5.8±1.1%) than patients with stable or deteriorated GOs (7.3±1.2%), although ophthalmic and radiological parameters were not significantly different at the start of the study. In conclusion, an expanded Teff population may be associated with GO pathogenesis. Additionally, decreased Tregs in peripheral blood may predict a good clinical outcome.

Key words: Graves' ophthalmopathy, FoxP3, CD25, Regulatory T cell, Effector T cell

GRAVES’ DISEASE (GD) is an autoimmune disease that principally affects the thyroid, resulting in hyperthyroidism and an enlarged thyroid. Graves’ ophthalmopathy (GO) is an inflammatory manifestation localized to the orbital connective tissue that occurs in 25–50% of patients with GD [1, 2]. In many instances, particularly in the milder forms, the disease may remit or improve spontaneously. However, in some cases, GO is a progressive disease that considerably impairs quality of life, may threaten a patient’s sight, and for which only limited therapeutic options with variable effectiveness are available. The factors that predict an improvement or deterioration of the clinical course are not well known.

Regulatory T cells (Tregs) typically suppress immune responses. They function via negative costimulatory molecules, induction of anti-inflammatory signal transduction pathways in T cells and antigen-presenting cells, direct or indirect destruction of effector cells and antigen-presenting cells, and secretion of suppressive cytokines [3]. Tregs coexpress CD4 and CD25, and were originally identified in mice as pos-
sessing suppressive capabilities towards autoimmune disease [4-6]. The transcriptional repressor FoxP3 controls the development and functions of Tregs [7]. Recent studies suggested that dysregulation of Tregs is a major risk factor in the development of autoimmune diseases; however, the role of Tregs in the pathogenesis and clinical characteristics of GO are not well defined. A previous study reported that, in a single GO patient, Tregs were increased after successful rituximab treatment [8]. Additionally, CD4⁺ CD25⁺ effector T cells (Teffs), which express small amounts of FoxP3, were increased in GO patients compared with healthy controls [9]. These studies suggested that the delicate balance between Tregs and Teffs is important in the pathogenesis of GO.

In this study, we therefore investigated differences in the frequencies of Tregs and Teffs in GD with overt ophthalmopathy (GOs) compared with GD without ophthalmopathy (non-GOs) and healthy controls (HCs). We distinguished CD4⁺ CD25⁺ FoxP3⁺ T cells (Tregs) and CD4⁺ CD25⁻ FoxP3⁻ T cells (Teffs) in peripheral blood [10] using flow cytometry. Furthermore, we evaluated whether the frequencies of Tregs and Teffs in GOs were related to the Clinical Activity Score (CAS), magnetic resonance imaging (MRI) findings, NOSPECS score, or the clinical course of GOs.

Materials and Methods

Patients

This study conformed to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the Faculty of Medicine, Tottori University. All patients provided written informed consent prior to inclusion in the study. Additionally, this study has been enrolled in UMIN clinical trials registry, with the ID number UMIN000013854. Sixty-three patients with GD were included in the study: 35 GOs and 28 non-GOs (Fig. 1). Thirty patients with GOs (86%) and 25 patients with non-GOs (89%) were treated with methimazole (MMI), and no patients were treated with propylthiouracil. Patients who received thyroidectomy or radio iodine therapy were excluded. Fifteen healthy euthyroid patients without thyroid autoantibodies or other autoimmune diseases served as HCs. Diagnosis of GO was based on the European Group of Graves’ Orbitopathy consensus [11]. Thyroid-related hormones and antibodies were measured using standard commercially available kits. The clinical characteristics of the study groups are summarized in Table 1.

Ophthalmic and clinical course assessments

Ophthalmic assessment was performed at the entry to the study. Proptosis, diplopia and CAS were recorded using the modified European Group of Graves’ Orbitopathy patient form [11]. The seven-point CAS (spontaneous retrobulbar pain, pain on attempted eye movements, conjunctival hyperemia, eyelid redness, chemosis, swelling of the caruncle, and swelling of the eyelids) and NOSPECS score were evaluated as previously reported [12, 13]. A Hertel exophthalmometer (Keeler Instruments Inc., Broomall, PA, USA) was used to measure proptosis. Diplopia was estimated using a Hess chart.

The clinical course was evaluated in GOs who were available for follow-up for at least 24 weeks and had not received glucocorticoids for at least 24 weeks since the entry into the study. Improvement was defined as meeting at least three of the following measures: 1) reduction of at least 3 mm in eyelid width, 2) reduction...
T cell subsets in Graves’ ophthalmopathy

Flow cytometry
Peripheral blood mononuclear cells were sampled at the beginning of this study. The cells were washed in PBS and suspended in staining buffer with antihuman CD4-PerCP (BD Biosciences, Bedford, MA, USA) and CD25-APC (BD Biosciences) in the volume indicated by the manufacturer. The cells were washed, resuspended in 1 mL of cold fixation/permeabilization buffer, and incubated at 4°C for 1 h (FoxP3 staining buffer set; eBioscience, San Diego, CA, USA). To analyze Tregs, the cells were stained with antihuman FoxP3-PE (eBioscience) and the matched isotype control (mouse; eBioscience), incubated at 4°C for 30 min in the dark, washed with 2 mL of permeabilization buffer, and resuspended in staining buffer for analysis. Ten thousand events were collected on a FACSCalibur 500 flow cytometer (BD Biosciences), gated on lymphocytes and CD4. Data were analyzed with CellQuest software version 4.0.2 (BD Biosciences).

Statistical analysis
Statistical comparisons of clinical parameters (age, thyroid functions, dose of MMI and autoantibodies), and frequencies of T cell subsets in the GOs (improved, stable and deteriorated groups), non-GOs and HCs groups were performed using one-way ANOVA. Correlations between the frequencies of T cell subsets and GO severity (CAS and proptosis) were determined using nonparametric Spearman’s correlation. Categorical parameters including female sex, smoking habits, and MRI findings were compared using the χ² test. All statistical tests were performed using SPSS for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). P values of p < 0.05 were considered statistically significant.

Table 1 Background of patients at the study outset

|                     | GOs (n = 35) | Non-GOs (n = 28) | HCs (n = 15) | p value |
|---------------------|-------------|------------------|-------------|---------|
| Age, years (SD)     | 47.3 (±15.5) | 43.5 (±15.4)     | 36.8 (±9.2) | 0.11    |
| Female sex, n (%)   | 22 (63%)    | 22 (82%)         | 12 (80%)    | 0.075   |
| Smoking habit, n (%)| 9 (25%)     | 4 (14%)          | 2 (13%)     | 0.50    |
| Antithyroid drug †  | yes, n (%)  |                  |             |         |
| MMI dose (mg)       | 8.6 (5.9)   | 7.9 (5.6)        | 0           | 0.62    |
| TSH (µIU/mL)        | 1.46 (2.1)  | 0.76 (1.0)       | 1.51 (0.54) | 0.22    |
| FT4 (ng/dL)         | 1.58 (0.91) | 1.80 (0.16)      | 1.22 (0.19) | 0.12    |
| FT3 (pg/mL)         | 6.50 (8.09) | 6.75 (6.28)      | 3.04 (0.25) | 0.09    |
| TRAb3rd (IU/L)      | 21.6 (23.5) | 14.1 (33.2)      | 0.38 (0.20) | 0.06    |
| TSAb (%)            | 1,328 (1,206) | 1,214 (1,453)    | 99 (10.1)  | 0.007 * |
| TPO Ab (IU/mL)      | 53.6 (80.8) | 168 (197.2)      | 8.9 (2.53)  | 0.002 * |
| Tg Ab (IU/mL)       | 395 (1,118) | 121 (178.9)      | 13 (3.3)   | 0.39    |

Date given as median (SD). † MMI was used for antithyroid medication. GOs, Graves’ disease with ophthalmopathy; Non-GOs, Graves’ disease without ophthalmopathy; HCs, Healthy controls; TRAb, TSH receptor antibody; TSAb, Thyroid stimulation antibody. * P-values are indicated for the differences among each group.
### Table 2 Clinical background and thyroid status of improved versus stable or deteriorated Graves’ ophthalmopathy patients

|                      | Improved (n = 14) | Stable or deteriorated (n = 21) | p value |
|----------------------|-------------------|--------------------------------|---------|
| Age, years ‡         | 44.1 (16.9)       | 49.4 (14.5)                    | 0.33    |
| Female gender, n (%) | 10 (71%)          | 12 (57%)                       | 0.39    |
| Smoker, n (%)        | 3 (21%)           | 6 (28%)                        | 0.64    |
| GO duration at study entry, months ‡ | 9.4 (4.3) | 10.2 (4.9) | 0.64 |
| Prior glucocorticoid therapy, n (%) | 7 (50%) | 12 (57%) | 0.68 |
| Prior radiotherapy, n (%) | 6 (43%) | 10 (48%) | 1.00 |
| **Anti-thyroid drugs,** |                |                                |         |
| yes; n (%)           | 12 (86%)          | 18 (86%)                       | 1.00    |
| dose (mg) ‡          | 6.6 (4.3)         | 10 (6.5)                       | 0.09    |
| TSH, µIU/mL          |                   |                                |         |
| at study outset ‡    | 2.10 (2.7)        | 1.04 (1.6)                     | 0.14    |
| at end of follow up ‡| 1.43 (1.6)        | 1.24 (1.4)                     | 0.82    |
| FT4, ng/dL           |                   |                                |         |
| at study outset ‡    | 1.63 (1.21)       | 1.53 (0.62)                    | 0.67    |
| at end of follow up ‡| 1.38 (0.41)       | 1.44 (0.51)                    | 0.69    |
| FT3, pg/mL           |                   |                                |         |
| at study outset ‡    | 6.09 (6.23)       | 6.83 (9.66)                    | 0.28    |
| at end of follow up ‡| 3.42 (1.5)        | 3.61 (1.3)                     | 0.41    |
| TRAb3rd, IU/L        |                   |                                |         |
| at study outset ‡    | 25.5 (32.1)       | 18.9 (15.3)                    | 0.46    |
| at end of follow up ‡| 11.9 (17.9)       | 9.7 (7.9)                      | 0.64    |
| TSAb, %              |                   |                                |         |
| at study outset ‡    | 958 (1,104)       | 1,601 (1,233)                  | 0.13    |
| at end of follow up ‡| 726 (462)         | 939 (761)                      | 0.37    |

‡ Date given as median (SD). Improved, Improved GO patients; Stable or Deteriorated, Stable or Deteriorated GO patients; GO, Graves’ ophthalmopathy; TRAb, TSH receptor antibody; TSAb, Thyroid stimulation antibody.

### Table 3 Ophthalmological and radiological parameters of improved versus stable or deteriorated Graves’ ophthalmopathy

|                      | Improved (n = 14) | Stable or Deteriorated (n = 21) | p value |
|----------------------|-------------------|--------------------------------|---------|
| CAS – mean (range)   |                   |                                |         |
| at study outset      | 2.5 (1–6)         | 2.8 (1–6)                      | 0.61    |
| at end of follow up  | 0.92 (0–2)        | 2.9 (0–6)                      | 0.001 * |
| CAS ≥ 3, n (%)       | 6 (43%)           | 7 (33%)                        | 0.57    |
| NOSPECS score        |                   |                                |         |
| Soft tissue involvement, n (%) 0/a/b/c | | | |
| at study outset      | 3 (21) / 4 (29) / 4 (29) / 3 (21) | 3 (14) / 8 (38) / 6 (29) / 4 (19) | 0.92    |
| at end of follow up  | 5 (36) / 5 (36) / 4 (29) / 0 (0)  | 1 (5) / 8 (38) / 7 (33) / 5 (24) | 0.044 * |
| Proposis, rt./lt. mm ‡ |                 |                                |         |
| at study outset      | 18.2 (2.8) / 18.6 (3.0) | 19.3 (2.9) / 18.9 (2.6) | 0.31, 0.78 |
| at end of follow up  | 17.8 (2.1) / 18.2 (1.9) | 19.7 (2.5) / 19.3 (2.6) | 0.030 *, 0.10 |
| Eye movement - diplopia, n (%), 0/a/b/c | | | |
| at study outset      | 5 (36) / 3 (21) / 3 (21) / 3 (21) | 5 (21) / 6 (29) / 7 (33) / 3 (14) | 0.74    |
| at end of follow up  | 6 (43) / 4 (29) / 2 (14) / 2 (14) | 3 (14) / 5 (24) / 10 (48) / 3 (14) | 0.14    |
| Intracocular pressure rt./lt., mmHg ‡ | | | |
| at study outset      | 14.9 (2.7) / 17.5 (4.5) | 15.3 (2.7) / 14.2 (2.4) | 0.98 / 0.15 |
| at end of follow up  | 14.7 (3.1) / 15.3 (4.1) | 15.1 (2.5) / 14.6 (2.8) | 0.97 / 0.67 |
| High intensity on T2WI, n (%) | | | |
| at study outset      | 5 (36%)           | 7 (33%)                        | 0.88    |

‡ Date given as median (SD). Improved, Improved GO patients; Stable or Deteriorated, Stable or Deteriorated GO patients; GO, Graves’ ophthalmopathy; CAS, Clinical activity score. * P-values are indicated for the differences between Improved and Stable or Deteriorated.
Results

Intracellular immunostaining and flow cytometric analysis of CD25 and FoxP3 expression

Dot plots of the flow cytometric analysis for representative cases are shown in Fig. 2. The upper left quadrant of each panel is FoxP3-negative and CD25-positive (Teffs). The upper right quadrant of each panel is FoxP3-positive and CD25-positive (Tregs). As shown in Fig. 2A and 2B, Teffs in GOs were increased compared with the non-GOs and HC groups (Fig. 2C and 2D respectively). Furthermore, Tregs in GOs (Fig. 2A and B) were also different between cases, depending upon the clinical course.

The frequency of Teffs (CD4⁴ CD25⁺ FoxP3⁻ cells) was significantly increased in GOs (30.8 ± 8.4%) compared with non-GOs (19.4 ± 7.1%, p < 0.001) and HCs (22.8 ± 7.9%, p = 0.004) (Fig. 3A). However, the frequency of Tregs was not significantly different between GOs (6.7 ± 1.4), non-GOs (6.8 ± 1.5) and HCs (6.8 ± 1.3) groups (Fig. 3B). No significant differences were observed between the proportion of FoxP3 positive and negative CD25 cells.

To evaluate factors affecting the CD4⁺ T cell subsets, we compared frequencies of CD4⁺ T cell subsets with thyroid function (hypothyroid, euthyroid, and hyperthyroid), the use of antithyroid drugs (yes or no), sex, age, and smoking habits. The frequencies of Teffs were significantly increased in male patients (30.6 ± 8.4%) compared with female patients (24.5 ± 9.6%, p = 0.023). However, the other parameters showed no differences in levels of Tregs and Teffs (data not shown).

Correlations between the subsets of CD4⁺ T cells and clinical parameters in Graves' ophthalmopathy

We next determined whether the frequencies of Tregs and Teffs correlated with disease activity, according to CAS and MRI findings, and disease severity according to NOSPECS score. The frequencies of Tregs and Teffs were not correlated with the CAS (r = 0.16 and 0.28, respectively). There were 13 patients with CAS ≥3 regarded as active GO and 22 patients with CAS <3 regarded as non-active GO. The frequencies of Tregs and Teffs did not show differences between CAS ≥3 and <3 (CAS≥3/<3 group: Tregs 6.64/6.70, p = 0.898; Teffs 32.7/28.5, p = 0.137). Similarly, the frequencies of Tregs and Teffs were not correlated with MRI findings (high-intensity/normal-intensity group on T2WI: Tregs 6.34/ 6.84, p = 0.374; Teffs 32.2/ 29.4, p = 0.360) nor NOSPECS score (soft tissue: Tregs, 0/a/b/c, 6.44/ 6.95/ 6.86/ 6.75; Teffs, 0/a/b/c, 33.6/ 31.3/ 25.6/ 34.4; proptosis (rt./lt.): Tregs r = 0.17/0.08, Teffs r = −0.01/−0.05; diplopia: Tregs, 0/a/b/c, 32.5/26.0/32.3/32.4).

Correlation between Tregs and clinical outcome in Graves' ophthalmopathy

Because the subsets of CD4⁺ T cells were not related to any parameters of GO activity and severity, we evaluated their relationship with the clinical outcome of GO 24 weeks after the initial measurement. We divided the patients into an improved GO group and a stable or deteriorated GO group according to the criteria described in the Materials and Methods section. The patients’ clinical characteristics including age, sex, smoking habits, prior steroid therapy, radiotherapy, use of MMI, thyroid function, thyroid antibodies, and duration of observation were not significantly different between the groups. As expected, at the end of follow-up, CAS and NOSPECS score were significantly different between the groups (Table 3). The ophthalmological and radiological parameters of each group are summarized in Table 3.

As shown in Fig. 2A and 2B, the proportion of CD25⁺ cells, especially the proportion of FoxP3⁺ cells (Tregs), decreased in improved GO compared with stable or deteriorated GOs. As shown in Fig. 3C, the Teff frequency in the improved GO group (27.8 ± 8.5%) was significantly higher than that of non-GOs (19.4 ± 7.1%, p = 0.01). The Teff frequency in the stable or deteriorated GO group (32.8 ± 8.0) was also significantly higher than that in HCs (22.8 ± 7.9%, p = 0.001) and non-GOs (19.4 ± 7.1%, p < 0.001). There were no significant differences in the Teff frequency between the improved GO and stable or deteriorated GO groups.

The Treg frequency was not significantly different among GOs, non-GOs and HCs (Fig. 3B). However, when we subdivided GOs by the clinical outcome, as shown in Fig. 3D, the Treg frequency was higher in the stable or deteriorated GOs compared with the improved GOs (improved group: 5.75 ± 1.12%; stable or deteriorated: 7.28 ± 1.2%, p = 0.0063).
Fig. 2 Flow cytometric analysis of CD4, CD25, and FoxP3 expression in peripheral blood mononuclear cells

Shown are dot plots from an improved Graves’ ophthalmopathy patient (GO) (A), a deteriorated GO patient (B), a Graves’ disease patient (non-GO) (C) and a healthy control (D). T cell subsets were identified by gating first on lymphocytes, followed by CD4, CD25, and FoxP3. CD4+ CD25+ FoxP3+ regulatory T cells (Tregs) are shown in the upper right quadrant, and CD4+ CD25− FoxP3− effector T cells (Teffs) are shown in the upper left quadrant.
Fig. 3  Frequencies of CD4⁺ T cell subsets in the peripheral blood of Graves’ ophthalmopathy patients (GOs), Graves’ disease patients without ophthalmopathy (non-GOs) and healthy controls (HCs)

The percentages (mean ± SD) of effector T cells (Teffs) (A) and regulatory T cells (Tregs) (B) among GOs (n = 35), non-GOs (n = 28) and HCs (n = 15) are shown in A and B, respectively. GOs were subdivided into an improved group (n = 14) and a stable or deteriorated group (n = 21) according to clinical outcome. The percentages of Teff (C) and Treg (D) cells in these groups are shown in C and D, respectively. Groups were statistically compared using ANOVA with the p-values indicated in the figure.
Discussion

Previous reports attempted to describe the role of Tregs and Teffs in the pathogenesis of GO [8, 9, 14] however, their exact contribution remained unclear. This study described an increased prevalence of circulating Teffs in GOs compared with non-GOs and HCs. Furthermore, GOs with a lower frequency of Tregs were predictive of a better prognosis.

The roles of T cells and cytokines in GO and GD have been establishing. For example, Th17 cells which is functionally the opposite subset of Tregs might be involved in GD pathogenesis [15, 16]. In addition, IL-17 which is produced by Th17 cells is a potent pro-inflammatory cytokine, and has been shown to increase in serum of GO patients [17, 18]. On the other hand, inflammatory cytokine, and has been shown to increase in serum of GO patients [17, 18]. On the other hand, on the other hand, CD4+ CD25+ FoxP3- cells, referred as Teffs in this study, have not been well established. To our knowledge, there are few reports which discuss CD4+ CD25+ FoxP3+ T cell subsets in GO. In tumor-infiltrating lymphocytes (TILs), not PBMCs, Ronald et al. reported the characteristics of CD4+ CD25+ FoxP3+ T cell subsets, including cytokine production, in the setting of primary high-grade ovarian cancer [19]. CD4+ CD25+ FoxP3+ T cells of TILs were thought to be Th1 cells because of their expression of cell surface marker such as GITR, CTLA4 and OX40. However, these cells failed to produce T-helper cytokines and expressed high levels of the exhaustion marker PD-1. These results suggested CD4+ CD25+ FoxP3+ TILs were functionally exhausted Th1 cells which might reflect ongoing recognition of tumor antigens. As a previous report about CD4+ CD25+ FoxP3+ T cell subsets in GO, Douglas et al. reported that CD4+ CD25+ T cells that expressed low levels of FoxP3 were increased in euthyroid GO patients compared with HCs [9]. Additionally, we observed an increased Teffs in GOs, not only compared with HCs, but also with non-GOs. Therefore, considering the characteristics of CD4+ CD25+ FoxP3+ T cells, and predominance of Th1 cells in GO [20, 21], and higher frequencies of Teffs in GOs compared with non-GOs, our results might reflect the Th1 response of GO being exhausted due to ongoing recognition of orbital antigens. Our data suggest that the high frequency of Teffs might be one of the factors precipitating the onset of orbital inflammation in GO.

Another major finding in our study was that GOs with a higher Treg frequency tended to display a decline in clinical course. The prevalence of Tregs was significantly different between the improved group and the stable or deteriorated group. Previous reports indicated that the frequency of Tregs was not different between GD and HCs [22] and GO and HCs [14]. Similarly, in our study, the Treg frequencies were not significantly different amongst GOs, non-GOs and HCs, and as previously reported [9]. However, when we determined whether the clinical outcome of GO was improved, stable or deteriorated, higher Treg frequency suggested worse clinical prognosis. Recently, the role of Tregs in various autoimmune diseases was further elucidated. In multiple sclerosis, the number of Tregs has been found to be increased in the cerebrospinal fluid compared with the peripheral blood [23, 24]. Moreover, demonstrating that Treg frequency and/or function was enhanced compared with normal controls, as per a previous report on rheumatoid arthritis [3], suggests a compensatory attempt to overcome the autoimmune disease by increasing Treg activity. Similarly, Treg frequency was also positively correlated with the systemic lupus erythematosus activity score [25]. These results suggest that increased Treg frequency could reflect the response to inflammation in autoimmune disease. In regards to the association of GO and Tregs, higher mRNA levels for FoxP3 were found in the orbital tissue of GO versus healthy controls, and in severe GO as compared to mild GO [26]. Cytotoxic T lymphocyte antigen 4 (CTLA4), plays a key role as a negative regulator of T cell activation [27] and is associated with the pathogenesis of GO [28-30]. In orbital tissue of severe GO, mRNA for CTLA4 was decreased compared with mild GO, while mRNA FoxP3 was increased [26]. This contradictory expression of FoxP3 and CTLA4 suggests the dysfunction of FoxP3 in severe GO. These results suggest that frequencies and function of Tregs are associated with GO. In our present study, we could not elucidate the pathogenesis of GO because our samples were PBMCs, not orbital tissue from GO patients, and we did not evaluate CTLA-4. However, our present results suggest that increased Tregs frequency could reflect the earlier response to orbit inflammation in GO. Because frequencies of Tregs were different according to their final prognosis although there was no difference of severity between each group at the outset of our study.

We analyzed additional factors that might affect the clinical outcome of GO, namely smoking habit, thyroid function, and thyroid-stimulating antibodies [31].
Smoking habit, in particular, plays an important role in the occurrence of GO and is also associated with a higher degree of severity and lower effectiveness of medical treatments [32]. However, in our study, these parameters showed no significant difference between improved GO patients and patients with stable or deteriorated disease.

A limitation of this study was that the design was observational. We could not clearly explain the mechanism for the differential frequencies of Teffs and Tregs according to the clinical course of GO patients. Additionally, our study contained only a small number of patients in each group and various clinical stages in GO. Therefore, a prospective study evaluating a large number of patients at the same clinical stage is necessary to definitively elucidate the role of Teffs and Tregs in GO. Also, we should evaluate the expression of PD1 in Teffs to make it clear why frequencies of Teffs increase in GO. Furthermore, we should evaluate Th17 cells to clarify the association between T cell subsets, including Treg, and GO pathogenesis.

We conclude that the Teff frequency in peripheral blood may be associated with the pathogenesis of GO. Furthermore, our results suggest that the Treg frequency in peripheral blood in GO may be a predictive marker of its clinical course.

Acknowledgments

This work was supported by the ‘Tottori University Hospital funds for exploratory research’.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

References

1. Bahn RS (2010) Graves’ ophthalmopathy. N Engl J Med 362: 726-738.
2. Stan MN, Garrity JA, Bahn RS (2012) The evaluation and treatment of Graves’ ophthalmopathy. Med Clin North Am 96: 311-328.
3. Brusko TM, Putnam AL, Bluestone JA (2008) Human regulatory T cells: Role in autoimmune disease and therapeutic opportunities. Immunol Rev 223: 371-390.
4. Nishizuka Y, Sakakura T (1969) Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. Science 166: 753-755.
5. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155: 1151-1164.
6. Asano M, Toda M, Sakaguchi S, Sakaguchi N (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 184: 387-396.
7. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF (2001) Scurfin (FoxP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem 276: 37672-37679.
8. Khanna D, Chong KK, Afifiya NF, Hwang CJ, Lee DK, et al. (2010) Rituximab treatment of patients with severe, corticosteroid-resistant thyroid-associated ophthalmopathy. Ophthalmology 117: 133-139.
9. Douglas RS, Gianoukakis AG, Goldberg RA, Kamat S, Smith TJ (2007) Circulating mononuclear cells from euthyroid patients with thyroid-associated ophthalmopathy exhibit characteristic phenotypes. Clin Exp Immunol 148: 64-71.
10. Tone M, Tone Y, Adams E, Yates SF, Frewin MR, et al. (2003) Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. Proc Natl Acad Sci USA 100: 15059-15064.
11. Bartalena L, Baldeschi L, Dickinson A, Eckstein A, Kendall-Taylor P, et al. (2008) Consensus statement of the European Group on Graves’ orbitopathy (EUGOGO) on management of GO. Eur J Endocrinol 158: 273-285.
12. Mourits MP, Prummel MF, Wiersinga WM, Koornneef L (1997) Clinical activity score as a guide in the management of patients with Graves’ ophthalmopathy. Clin Endocrinol (Oxf) 47: 9-14.
13. Werner SC (1977) Modification of the classification of the eye changes of Graves’ disease: recommendations of the Ad Hoc Committee of the American Thyroid Association. J Clin Endocrinol Metab 44: 203-204.
14. Kahaly GJ, Shimony O, Gellman YN, Lytton SD, Eshkar-Sebban L, et al. (2011) Regulatory T-cells in Graves’ Orbitopathy: Baseline Findings and Immunomodulation by Anti-T Lymphocyte Globulin. J Clin Endocrinol Metab 96: 422-429.
15. Bedoya, Lam B, Lau K, Larkin J 3rd (2013) Th17 cells in immunity and autoimmunity. Clin Dev Immunol 2013: 986789.
16. Nanba T, Watanabe M, Inoue N, Iwatani Y (2009)
Increase of the Th1/Th2 cell ratio in severe Hashimoto’s disease and in the proportion of Th17 cells in intractable Graves’ disease. *Thyroid* 19: 495-501.

17. Wei H, Guan M, Qin Y, Xie C, Fu X, *et al.* (2014) Circulating levels of miR-146a and IL-17 are significantly correlated with the clinical activity of Graves’ ophthalmopathy. *Endocr J* 61: 1087-1092.

18. Kim SE, Yoon JS, Kim KH, Lee SY (2012) Increased serum interleukin-17 in Graves’ ophthalmopathy. *Graefes Arch Clin Exp Ophthalmol* 250: 1521-1526.

19. DeLeeuw RJ, Kroeger DR, Kost SE, Chang PP, Webb JR, *et al.* (2015) CD25 identifies a subset of CD4<sup>+</sup>FoxP3<sup>+</sup> TILs that are exhausted yet prognostically favorable in human ovarian cancer. *Cancer Immunol Res* 3: 245-253.

20. Gianoukakis AG, Khadavi N, Smith TJ (2008) Cytokines, Graves’ Disease, and Thyroid-Associated Ophthalmopathy. *Thyroid* 18: 953-958.

21. Aniszewski JP, Valyasevi RW, Bahn RS (2000) Relationship between disease duration and predominant orbital T cell subset in Graves’ ophthalmopathy. *J Clin Endocrinol Metab* 85: 776-780.

22. Pan D, Shin YH, Gopalakrishnan G, Hennessey J, De Groot LJ, *et al.* (2009) Regulatory T cells in Graves’ disease. *Clin Endocrinol (Oxf)* 71: 587-593.

23. Feger U, Luther C, Poeschel S, Melms A, Tolosa E, *et al.* (2007) Increased frequency of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. *Clin Exp Immunol* 147: 412-418.

24. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, *et al.* (2008) Compromised CD4<sup>+</sup>CD25<sup>high</sup> regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* 123: 79-89.

25. Lin SC, Chen KH, Lin CH, Kuo CC, Ling QD, *et al.* (2007) The quantitative analysis of peripheral blood FoxP3-expression T cells in systemic lupus erythematosus and rheumatoid arthritis patients. *Eur J Clin Invest* 37: 987-996.

26. Pawlowski P, Wawrusiewicz-Kurylonek N, Eckstein A, Reszec J, Luczynski W, *et al.* (2015) Disturbances of modulating molecules (FOXP3, CTLA-4/CD28/B7, and CD40/CD40L) mRNA expression in the orbital tissue from patients with severe Graves’ ophthalmopathy. *Mediators Inflamm* 2015: 340934.

27. Bayry J (2009) Autoimmunity: CTLA-4: a key protein in autoimmunity. *Nat Rev Rheumatol* 5: 244-245.

28. Vaidya B, Imrie H, Perros P, Dickinson J, McCarthy M, *et al.* (1999) Cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphism confers susceptibility to thyroid associated orbitopathy. *Lancet* 354: 743-744.

29. Villanueva R, Inzerillo AM, Tomer Y, Barbesino G, Meltzer M, *et al.* (2000) Limited genetic susceptibility to severe graves’ ophthalmopathy: no role for CTLA-4 but evidence for an environmental etiology. *Thyroid* 10: 791-798.

30. Borodic G, Hinkle DM, Cia Y (2011) Drug-induced graves disease from CTLA-4 receptor suppression. *Ophthal Plast Reconstr Surg* 27: e87-e88.

31. Marius NS, Bahn RS (2010) Risk factors for development or deterioration of Graves’ ophthalmopathy. *Thyroid* 20: 777-783.

32. Hagg E, Asplund K (1987) Is endocrine ophthalmopathy related to smoking? *Br Med J (Clin Res Ed)* 295: 634-635.