The proportion of peripheral blood Tregs among the CD4+ T cells of autoimmune thyroid disease patients: a meta-analysis

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Abstract. Autoimmune thyroid disease (AITD) is characterized by a loss of self-tolerance to thyroid antigen. Tregs, whose proportions are controversial among CD4+ T cells from AITD patients (AITDs), are crucial in immune tolerance. Considering that drugs might affect Treg levels, we assumed that the differences originated from different treatment statuses. Thus, we performed a meta-analysis to explore proportions of Tregs in untreated and treated AITDs. PubMed, Embase and ISI Web of Knowledge were searched for relevant studies. Review Manager 5.3 and Stata 14.0 were used to conduct the meta-analysis. Subgroup analysis based on different diseases and cell surface markers was performed. Egger linear regression analysis was used to assess publication bias. Approximately 1,100 AITDs and healthy controls (HCs) from fourteen studies were included. Proportions of Tregs among CD4+ T cells of untreated AITDs were significantly lower than those in HCs (p = 0.002), but were not in treated patients (p = 0.40). Subgroup analysis revealed lower proportions of Tregs in untreated Graves’ disease patients (GDs) (p = 0.001) but did not show obvious differences in untreated Hashimoto’s thyroiditis patients (HTs) (p = 0.62). Furthermore, proportions of circulating FoxP3+ Tregs were reduced in untreated GDs (p < 0.00001) and HTs (p = 0.04). No publication bias was found. In this first meta-analysis exploring proportions of circulating Tregs among CD4+ T cells of AITDs with different treatment statuses, we found that Tregs potentially contribute to the pathogenesis of AITD but function differently in GD and HT. Remarkably, FoxP3+ Tregs, which were decreased in both diseases, might be promising targets for novel therapies.

Key words: Regulatory T cells, Autoimmune thyroid diseases, Graves’ disease, Hashimoto’s thyroiditis, Meta-analysis
[25] might affect the differentiation and proliferation of Tregs through regulation of AMP-activated protein kinase (AMPK) [26] and signal transducer and activator of transcription 3 (STAT3) [27], we speculated that the origins of these differences between results lie in differences in the treatment statuses of the AITDs involved in the studies. Therefore, we performed a meta-analysis of the proportions of peripheral blood Tregs among CD4+ T cells of untreated and treated AITDs to explore how Tregs function in the pathogenesis of AITD.

Materials and Methods

Literature search

Three databases, including PubMed, Embase and ISI Web of Knowledge, were searched for relevant articles up to February 2019. The following search terms were used: “T Lymphocytes, Regulatory or Regulatory T Lymphocytes or Regulatory T Lymphocyte or T Lymphocyte, Regulatory or Regulatory T Cells or Regulatory T Cells or T Cells, Regulatory or Treg Cells or Cell, Treg or Cells, Treg or Treg Cell or Th3 Cells or Cell, Th3 or Cells, Th3 or Th3 Cell or Suppressor T Lymphocytes, Naturally Occurring or Naturally Occurring Suppressor T Lymphocyte or Naturally Occurring Suppressor T Lymphocyte or Naturally Occurring Suppressor T Lymphocytes or Suppressor T Lymphocytes, Naturally, Occurring or Suppressor T Lymphocyte, Naturally Occurring or Suppressor T Cells, Naturally Occurring or Naturally Occurring Suppressor T Cell or Naturally Occurring Suppressor T Cells or Suppressor T Cells, Naturally, Occurring or Suppressor T Cell, Naturally Occurring or T Cell, Naturally Occurring Suppressor or T Cells, Naturally Occurring Suppressor or Treg Cells or Cell, Treg1 or Cells, Treg1 or Treg1 Cell or “T Lymphocytes, Regulatory” [Mesh]” combined with “autoimmune thyroid disease or autoimmune thyroiditis or lymphocytic thyroiditis or Grave’s disease or Hashimoto’s thyroiditis or atrophic thyroiditis or postpartum thyroiditis or painless thyroiditis or silent thyroiditis or thyroiditis or Graves’ disease or thyroid or AITD or thyroid autoimmunity”. There were no limits regarding ethnicity or geographic region.

Inclusion criteria

Studies meeting all of the following criteria were included in the meta-analysis: (1) case-control study; (2) AITD diagnosed as primary AITD without chronic infectious disease; (3) treatment status of AITD reported; (4) Tregs assessed in peripheral blood in AITDs; (5) sufficient data provided, or data could be converted to the mean and standard deviation of the Tregs/CD4+ T cells in AITDs and HCs; and (6) published in English. There were no cases of overlapping studies. Studies using animal models or cell culture, reviews, case reports, and conference abstracts without subsequent publications were excluded from the analysis.

Data extraction and quality assessment

Data from the selected studies were extracted by two individual reviewers. The following data were extracted: first author’s name; publication year; country; specific type of AITD; Treg definition; treatment of the enrolled participants, and study results. The quality of the included studies was assessed by the Newcastle-Ottawa Scale (NOS), and studies that obtained scores of six or more were considered to be of high quality.

Statistical analysis

Review Manager 5.3 (Nordic Cochran Centre, Copenhagen, Denmark) and Stata 14.0 (Stata Corp. LP, College Station, TX) were used to conduct the meta-analysis. Heterogeneity was evaluated by the I² statistic. I² > 50% was considered to indicate a high degree of heterogeneity, and a random effects model was used to perform the analysis. For I² < 25%, a fixed effect model was utilized. Subgroup analysis based on different diseases and cell surface markers was implemented by Review Manager 5.3. To assess publication bias, Egger linear regression analysis was conducted by Stata 14.0.

Results

Selection and characteristics of the studies

The flow chart of the article search and inclusion process is displayed in Fig. 1. There were fourteen studies [11-24] included in the meta-analysis after screening 905, 516 and 1,997 potential articles obtained from PubMed, Embase and ISI Web of Knowledge, respectively. The characteristics of the fourteen studies as well as their NOS scores are summarized in Table 1. Among them, ten studies [12, 14, 15, 18-24] involved untreated AITDs, while eight studies [11, 13, 15-18, 20, 21] referred to treated patients. The number of studies focusing on GD and HT was ten and nine, respectively, while only one involved chronic autoimmune thyroiditis (CAT) [19]. The NOS scores of all of the studies were ≥6 (Table 1).

Meta-analysis of the peripheral blood Tregs in untreated and treated AITDs compared to HCs

Because treatment status might affect the level of Tregs, we performed a meta-analysis in untreated and treated AITDs. Among the ten studies of untreated AITDs, seven reported significantly decreased proportions of circulating Tregs in AITDs compared to HCs [12, 14, 15, 18, 20-22], one displayed nonsignificantly lower proportions of Tregs [19], and two studies revealed
increased proportions of Tregs [13, 14]. Because of the high degree of heterogeneity according to the $I^2$ statistics ($I^2 = 94% > 50%$), we utilized a random effects model in the subsequent meta-analysis. Compared with those in HCs, the proportions of circulating Tregs in untreated AITDs were clearly diminished ($-1.20 [-1.97, -0.43], p = 0.002$). Regarding treated AITDs, a total of eight studies were included, of which five studies suggested that the proportions of circulating Tregs were lower in these patients than in HCs, but the differences were not significant [13, 15, 17, 18, 20], one study reported an obvious decrease [16], and the other reported a slight increase [14]. As a result of the high degree of heterogeneity ($I^2 = 64% > 50%$), a random effects model was used, and no significant difference was found ($-0.16 [-0.52, 0.21], p = 0.40$). Forest plots of these analyses are shown in Fig. 2. Of note, three included studies simultaneously applied disparate definitions of Tregs. Among them, two studies suggested that different markers for the assessment of Tregs yielded a similar conclusion [15, 24], while the other one did not [11]. No difference was observed after comparing these two differently defined groups with HCs in that study respectively [11]. ($p = 0.36$ & $p = 0.40$). Considering that a high degree of heterogeneity might originate from different types of AITD and different cell surface markers, subgroup analysis was conducted.

**Subgroup analyses and publication bias**

To explore the sources of the high heterogeneity, we first performed subgroup analysis based on different AITD types. Three diseases, including GD, HT and CAT, were represented among the included studies, although only one study focused on CAT. Considering that CAT and HT can be classified together, we analyzed studies involving GD and HT (including CAT) (Fig. 3). After analyzing six heterogeneous studies involving untreated GD ($I^2 = 90% > 50%$) and another seven heterogeneous
Subsequently, we analyzed studies in which Tregs were defined as “FoxP3+” cells in untreated GD and HT patients (GDs and HTs) to explore potential reasons for the high heterogeneity (Fig. 4). The $I^2$ statistic suggested that the three studies on untreated GDs that defined Tregs as “FoxP3+” were homogeneous ($I^2 = 0$%), which implied that different cell surface markers might lead to heterogeneity. Remarkably, the proportions of circulating FoxP3+ Tregs were significantly lower in the untreated GDs ($p < 0.00001$) and HTs ($p = 0.04$). Because only two studies defined Tregs as CD4+ CD25+ and CD4+ CD25high in treated HTs, we did not perform a subgroup analysis similar to that performed in untreated patients.

### Table 1 Characteristics of the studies included in the meta-analysis.

| Author       | Region | AITD type | Treg definition | Treatment status | Case (GD/HT) | HC | Tregs/CD4+ T cells ratio in Cases (mean ± SD, %) | Tregs/CD4+ T cells ratio in Control (mean ± SD, %) | NOS score |
|--------------|--------|-----------|-----------------|------------------|-------------|----|-----------------------------------------------|-----------------------------------------------|-----------|
| Afeltra, A   | Rome   | GD        | CD4+ CD45RA+     | All patients untreated | 15 (15/0) | 25 | 43.85 ± 13.5% | 52.3 ± 6.5% | 8         |
| Fountoulakis, S | Greece | HT GD    | CD4+ CD25+ CD4+ CD25high | All patients untreated | 70 (15/55) | 20 | 53.99 ± 10.21 | 49.6 ± 8.2% | 9         |
| Glick A B    | America| HT GD    | CD4+ CD25high    | All patients untreated | 20 (7/13) | 9  | 6.1 ± 0.4% | 6.5 ± 0.4% | 8         |
| Klatka, M    | Poland | GD        | CD4+ CD25+ Foxp3+ | All patients untreated | 60 (60/0) | 20 | 3.76 ± 1.92% | 7.35 ± 1.99% | 8         |
| Li, C        | China  | GD HT    | CD4+ FoxP3+      | All patients untreated | 31 (16/15) | 12 | 0.814 ± 0.256% | 1.644 ± 0.544% | 6         |
| Liu, Y       | China  | HT        | CD4+ CD25+ CD127low | All patients untreated | 25 (0/25) | 20 | 0.322 ± 1.458% | 5.298 ± 1.600% | 6         |
| Mao, Chaoming | China  | GD HT    | CD4+ CD25+ FOXP3+ CD4+ CD25high Foxp3+ | Some patients treated | 68 (58/10) | 48 | 2.001 ± 1.006% | 3.36 ± 0.96% | 7         |
| Matsuzawa, K | Japan  | GD        | CD4+ CD25+ FoxP3+ | All patients treated | 63 (63/0) | 12 | 6.74 ± 1.434% | 6.8 ± 1.3% | 7         |
| Nakano, A    | Japan  | GD HT    | CD4+ CD25+ CD69+ CD4+ CD25+ Foxp3+ | All patients treated | 13 (NR) | 11 | 14.09 ± 7.460 | 13.27 ± 5.706% | 7         |
| Paggi, A     | Italy  | GD        | CD4+ CD45RA+     | All patients treated | 11 (11/0) | 20 | 36.1 ± 14.7% | 47.9 ± 14.7% | 7         |
| Roman        | Colombia | GD HT    | CD4+ CD25high    | All patients treated | 28 (13/15) | 16 | 5.767 ± 2.914% | 7.214 ± 4.21% | 7         |
| Siklar, Z    | Turkey | CAT       | CD4+ CD25+ Foxp3+ | All patients untreated | 32 (0/0) | 24 | 3.6 ± 0.637% | 3.8 ± 0.637% | 8         |
| Watanabe, M  | Japan  | HT        | CD4+ CD25+       | Some patients treated | 93 (0/93) | 57 | 58.72 ± 12.14% | 52.3 ± 10.5% | 7         |
| Xue, H       | China  | HT        | CD4+ CD25highFoxp3+ | All patients untreated | 48 (0/48) | 32 | 1.64 ± 0.49% | 3.90 ± 1.36% | 6         |

AITD, Autoimmune thyroid disease; GD, Graves Disease; HT, Hashimoto Thyroiditis; HC, Healthy Control; Treg, Regulatory T cell; CAT, Chronic autoimmune thyroiditis.
Egger linear regression analysis was utilized to evaluate publication bias, and no bias was found in the untreated ($p = 0.065$) and treated ($p = 0.072$) AITDs (Fig. 5).

Additional meta-analysis

Considering that there was no consensus about whether different thyroid functions affect the proportions of circulating Tregs in HTs, we rechecked the potentially eligible articles and found one additional study [28].

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**Fig. 2** Forest plot of the differences in the proportions of peripheral blood Tregs among the CD4+ T cells in (A) untreated AITDs and (B) treated AITDs.

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**Fig. 3** Forest plots of the differences in the proportions of peripheral blood Tregs among the CD4+ T cells in untreated patients based on different AITD types, including GD (top) and HT (bottom).
proportion of circulating Tregs tended to be lower in subclinical hypothyroid \( (p = 0.74) \) and euthyroid \( (p = 0.59) \) HTs than in HCs, but the same trend was not found in hypothyroid HTs \( (p = 0.93) \) (Fig. 6).

**Discussion**

In this study, the proportion of circulating Tregs among the CD4+ T cells of untreated and treated AITDs was investigated by meta-analytical methods. To our knowledge, this is the first meta-analysis exploring the proportions of circulating Tregs among the CD4+ T cells of AITDs with different treatment statuses. Consistent with analysis of Tregs in other autoimmune diseases [29], our analysis revealed that the proportions of circulating Tregs tended to be lower in untreated and treated AITDs, which implied that Tregs potentially contribute to the pathogenesis of AITD.

Our results indicated that the proportion of circulating Tregs among the CD4+ T cells of untreated AITDs was lower than in HCAs, but no significant difference was found in treated AITDs. Because of the small number of studies focusing on changes in the proportion of Tregs before and after treatment in AITDs, a conclusion that the proportion of Tregs increased following treatment, as has been reported for both systemic sclerosis [30] and pulmonary tuberculosis patients [31], could not be drawn. More studies aiming to clarify the correlation between changes in Tregs and treatment are expected.

Subgroup analysis based on different types of AITD indicated that the proportion of circulating Tregs was lower in untreated GDs than in HCs, which is in accordance with previous studies that revealed a significantly lower percentage of Tregs in female rhesus monkeys with GD [32]. When Tregs were depleted, both disease incidence in resistant C57BL/6 mice and disease severity in susceptible BALB/c mice were enhanced [33]. Thus, reduced proportions of circulating Tregs potentially contribute to the development of GD.

With respect to HT, no significant difference in the untreated and treated groups compared to HCs was found, which implies that Treg dysfunction exists to some extent. Indeed, a significant reduction in the level of suppression of proliferation was observed in HTs after coculture of Tregs and Teffs [16]. Additionally, the syn-
thesis of IL-1β and TNF-α was not significantly inhibited when the suppressive activity of Tr1 cells on cytokine release was analyzed [34]. The results of our additional meta-analyses as well as increased \(\text{FOXP3} \) mRNA expression in T cells in hypothyroid HTs [35] indicate that the level of Tregs in HTs is associated with thyroid function, which has not been clarified in GD. All of the above findings suggest that in contrast with GD, dysfunction rather than a decreased proportion of Tregs dominates in HT.

The transcription factor FoxP3, whose expression level is essential for the function of Tregs, supports the maintenance of an immunosuppressive milieu [36]. The results of subgroup analysis in untreated GDs and HTs based on different cell surface markers revealed a low degree of heterogeneity in the former group, which indi-
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Staten a possible origin of heterogeneity. Remarkably, the proportions of circulating FoxP3+ Tregs decreased significantly in untreated GDs and HTs. Polymorphisms and haplotypes of the FOXP3 gene [37] as well as FoxP3 splice variants [38] were correlated with susceptibility to GD and HT. Zhang and colleagues revealed reduced expression of Foxp3 via upregulation of SIRT1 and RAR-related orphan receptor gamma t (RORγt) in GDs [39]. Likewise, the acetylation level and expression of FOXP3, mediated by SIRT1, were lower in HTs [9].

To summarize, FoxP3+ Tregs are clearly dysregulated in the development of GD and HT.

Some limitations existed in our meta-analysis. First, the number of AITDs included in our analysis was not sufficient, which partly attenuates the credibility. Second, we excluded some studies whose data were not adequate or available during the process of study selection, which was expected to weaken the strength of our results. Third, although different cell surface markers were used to define Tregs, there is no optimum way to precisely “capture” all of the Tregs in peripheral blood. In other words, only Tregs that could be recognized via methods used in the included studies were reflected in our results. Finally, the impact of treatment on Tregs was only partially confirmed due to the incomparability between untreated and treated AITDs.

In conclusion, the proportion of peripheral blood Tregs in CD4+ T cells tends to be lower in untreated and treated AITDs than in HCs. Of note, Foxp3+ Treg-based therapies have been used in preclinical and clinical trials in other autoimmune diseases that are also triggered by impaired Tregs [40], which sheds new light on AITD treatment. Undoubtedly, new studies will improve upon our present analysis. A larger sample size, better methods to define Tregs and a direct comparison between patients with different treatment statuses are expected to be achieved in a new study.

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Disclosure

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