Increased differentiation of Th22 cells in Hashimoto’s thyroiditis

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Abstract. As Th22 subsets are identified, their involvement in the pathogenesis of numerous autoimmune diseases has become apparent. In this study, we investigated differentiation of Th22 cells in the autoimmune thyroid diseases including Hashimoto’s thyroiditis (HT) and Graves’ disease (GD). Besides, we also explored the involvement of Th22 cells in an iodine-induced autoimmune thyroiditis (AIT) model (i.e., NOD.H-2H4 mice). In HT patients, we showed the level of circulating Th22 cells correlated with the level of serum IL-22, and was significantly higher than in GD patients and healthy control subjects. Levels of serum IL-6, a major Th22 cell differentiation effector, were also higher in HT, and correlated with Th22 cells concentration. Peripheral blood mononuclear cells isolated from HT patients produced larger amounts of IL-6 in vitro than did those isolated from other groups. Furthermore, unlike those from GD patients, T lymphocytes from HT patients showed an enhanced differentiation in vitro into Th22 cells in the presence of recombinant IL-6 and TNF-α. In addition, levels of circulating Th22 cells and titers of thyroid peroxidase antibody were positively correlated in HT patients. In NOD.H-2H4 mice, higher numbers of Th22 cells were observed in the spleens of the AIT group, while splenocytes of this group also produced larger amounts of IL-6 and IL-22 in vitro compared with the control. Intra-thyroid infiltrating IL-22+ lymphocytes were significantly increased in mice of the AIT group compared with the control. Our results indicate that Th22 cells may contribute to the pathogenesis of HT.

Key words: Hashimoto’s thyroiditis, Graves’ disease, Th22 cell, IL-6, IL-22

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Studies have shown that IL-22 is produced by new Th cells which do not express IL-17, IFN-γ, or IL-4 [17, 18]. The Th22 cell line primarily secretes IL-22 and TNF-α and expresses CC chemokine receptor (CCR) 4, CCR6, and CCR10. Th22 cell develops along a pathway that is distinct from that of the Th1, Th2, or Th17 differentiation pathways [17, 19]. To induce Th22 differentiation, IL-6 is a major effector, while it can also be promoted by TNF-α in the presence of IL-6. It was originally reported that Th22 cells were important in the pathogenesis of chronic inflammatory skin diseases [20, 21]. Th22 cells have also been shown to contribute to numerous autoimmune diseases such as atopic dermatitis [22], Behcet’s disease [19], systemic lupus erythematosus [23], rheumatoid arthritis [24], immune thrombocytopenia [25], systemic sclerosis [26], and others. Recently, it was reported that Th22 cells were elevated at the onset of GD [27] and in newly diagnosed HT patients [28].

Studies on Th22 cells in patients with AITD are limited. In this work, we assessed the differentiation of circulating Th22 cells and related cytokines in patients with AITD, established in vitro polarization of Th22 cells in Peripheral blood mononuclear cells (PBMCs) from subjects. Furthermore, the correlation of circulating Th22 cells and TPOAb titers was analyzed. Finally, we explored the role of Th22 cells in the iodine-induced AIT mode in NOD.H-2h4 mice.

Materials and Methods

Subjects and clinical assessment

The Institutional Ethics Committee of China Medical University approved the study. The research was performed in accordance with the tenets of the Declaration of Helsinki. Each participant provided written informed consent. Patients were recruited from First Affiliated Hospital of China Medical University. These included 35 HT patients afflicted with goiter, TPOAb titers higher than 100 IU/mL, in euthyroid state or subclinical hypothyroidism. They didn’t have replacement therapy. Subjects with GD in this study consisted of 24 patients with untreated GD (uGD) in enhanced thyroid function (serum FT3 >46.08 pmol/L, the upper limit of our detection), and 12 with euthyroid GD (eGD), having normal thyroid function ≥6 months due to methimazole therapy. In addition, 21 healthy individuals were enrolled as control subjects (Table 1).

Levels of serum FT4, FT3, TSH, TPOAb, and TGAb were measured by electrochemiluminescent immunoassay (Abbott, Table1). TRAb levels were measured by electrochemiluminescent immunoassay on a Cobas e601 analyzer (Roche).

Mice and iodine-induced thyroiditis

NOD.H-2h4 mice, obtained from Jackson Laboratory (Bar Harbor, ME, USA), were bred in the animal facility of China Medical University. All animal care and experimental procedures were performed in accordance with the Guideline for Animal Experimentation, with the approval of the Animal Ethics Committee of China Medical University.

Thirty male NOD.H-2h4 mice were randomly divided into two groups at 6 weeks of age: a control group was given sterile water, and an AIT group was given 0.05% sodium iodine (NaI) in the drinking water to induce thyroiditis. Twelve weeks after NaI provision, mice were euthanized, and the thyroid glands, blood, and spleens were harvested to determine the extent of thyroiditis, the serum TGAb titers, and cytokine concentrations.

Table 1 Clinical features of patients and healthy donors included in the study*

<table>
<thead>
<tr>
<th></th>
<th>HT</th>
<th>uGD</th>
<th>eGD</th>
<th>Control</th>
<th>Nominal range</th>
</tr>
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<tbody>
<tr>
<td>Sample size (n)</td>
<td>35</td>
<td>24</td>
<td>12</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>0/35</td>
<td>4/20</td>
<td>1/11</td>
<td>4/17</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>34±8</td>
<td>34±8</td>
<td>32±9</td>
<td>30±3</td>
<td></td>
</tr>
<tr>
<td>FT4 (pmol/L)</td>
<td>12.40 (9.56-17.47)</td>
<td>46.65 (31.09-77.22)</td>
<td>14.80 (11.23-17.05)</td>
<td>16.32 (12.32-18.37)</td>
<td>9.01-19.05</td>
</tr>
<tr>
<td>FT3 (pmol/L)</td>
<td>3.74 (2.81-5.22)</td>
<td>&gt;46.08</td>
<td>3.70 (2.93-5.12)</td>
<td>4.22 (3.03-5.31)</td>
<td>2.63-5.70</td>
</tr>
<tr>
<td>TSH (mIU/L)</td>
<td>5.82 (1.28-18.96)</td>
<td>0 (0-0.01)</td>
<td>3.13 (1.11-4.59)</td>
<td>1.87 (1.27-4.17)</td>
<td>0.35-4.94</td>
</tr>
<tr>
<td>TPOAb (IU/mL)</td>
<td>1200 (155.21-7250)</td>
<td>93.62 (1.04-1000)</td>
<td>24.75 (0.60-1000)</td>
<td>negative</td>
<td>0.11-5.23</td>
</tr>
<tr>
<td>TGAb (IU/mL)</td>
<td>73.17 (2.21-100)</td>
<td>27.14 (0.62-766.05)</td>
<td>32.32 (14.91-242.16)</td>
<td>negative</td>
<td>0.81-3.83</td>
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<tr>
<td>TRAb (IU/L)</td>
<td>-</td>
<td>24.46±11.70</td>
<td>0.49±0.43</td>
<td>0-1</td>
<td></td>
</tr>
</tbody>
</table>

*Data correspond to mean ± SD or median (range) according to the distribution. M, male; F, female. A “-” indicates that the experiment was not performed, or data is not applicable. HT, Hashimoto’s thyroiditis; uGD, untreated Graves’ disease; eGD, euthyroid Graves’ disease
Th22 cells in Hashimoto’s thyroiditis

GolgiPlug were added for another 5 h. Supernatants were collected before PMA and ionomycin stimulation for cytokine analysis via ELISA.

Flow cytometry analysis
To detect Th22 cells in PBMCs or splenocytes, cells were washed, immunostained with anti-CD4-FITC, fixed, permeabilized, and then serially incubated with intracellular cytokine antibodies including anti-IFN-γ, anti-IL-17A, and anti-IL-22. For detecting Th22 polarization, cells were immunostained with anti-hCD3-APC and anti-hCD8-FITC, then fixed, permeabilized, and incubated with IL-22-PE. Isotype-matched antibody controls were used in all procedures. Cells were analyzed with a FACSCalibur flow cytometer. Th22 cells were defined as CD4^+IFN-γ^-IL17-IL-22^+ cells.

Assessment of thyroiditis in mice
Thyroiditis in mice was evaluated as we previously reported [3]. Briefly, thyroid tissue sections (5-μm thick) were prepared and stained with hematoxylin and eosin (H&E). Severity of thyroiditis was graded on the following scale based on the approximate area of lymphocytic infiltration: 0 = normal; 1 = 1-10%; 2 = 10-30%; 3 = 30-50%; 4 > 50%.

Immunofluorescence and confocal microscopy
Thyroid tissue frozen sections (5.0 μm thick) were mounted on glass slides and stained by an immunofluorescence method. Briefly, sections were blocked with 1% BSA in PBS for 1 h, permeabilized in 0.3% Triton for 20 min, and Incubated for 2 h at room temperature with anti-mIL-22 mAbs diluted in PBS. After washed, the sections were incubated with PE-conjugated secondary antibody, followed by incubation with DAPI nuclear staining for 1 min. The images were obtained by a Leica SP5 confocal microscope under 40× air objective. Data are presented as the percentage of high IL-22-positive cells in lymphocytes.

ELISA
Sera and supernatants were stored at -20 °C until used. The hIL-22, hIL-6, mIL-22, and mIL-6 levels were measured in duplicate with ELISA kits (Dakewe Biotech), in accordance with the manufacturer’s instructions. Serum TGAb from mice were also mea-
Lymphocytes from HT patients showed higher rate of differentiation into Th22 cells in vitro

It has been reported that Th22 cells develop along a pathway that is distinct from that of the Th1, Th2, or Th17 cells differentiation pathways [17, 19]. For the present assay, freshly isolated PBMCs were cultured in the presence of anti-CD3, anti-CD28, anti-IFN-γ, and anti-IL-4 antibodies, and recombinant IL-2, IL-6, and TNF-α for Th22 cell polarization. We confirmed that CD4 T cells in PBMCs differentiated into large numbers of IL-22-producing CD4 T cells (CD3+CD8-IL-22+ cells) after the addition of IL-6 and TNF-α (Fig. 2B). PBMCs from HT patients differentiated into more polarized IL-22-producing CD4 T cells in vitro in the presence of IL-6 and TNF-α, compared with other groups, while the uGD and control groups were similar (Fig. 2B, 2D). In addition, in vitro polarized Th22 cells from HT patients produced significantly larger amounts of IL-22 compared with the other groups, as determined by ELISA of the supernatant (Fig. 2E).

Results

Percentage of circulating Th22 cells correlated with Th17 cells was increased in HT patients

Circulating CD4+IFN-γIL-17AIL-22+ cells as Th22 cells were measured (Fig. 1A, 1B). The proportion of peripheral Th22 cells in CD4+ T cells was significantly higher in patients with HT (2.07 ± 1.18%) than in the uGD (0.78 ± 0.48%, p < 0.001), eGD (0.67 ± 0.46%, p < 0.001), and healthy control (0.71% ± 0.42%, p < 0.001; Fig. 1C) groups. No differences were observed among the uGD, eGD, and control groups (Fig. 1C).

The proportion of peripheral Th17 cells in HT patients was also significantly higher than in other groups (Fig. 1D). Then, we analyzed the relation between the proportion of Th22 cells and Th17 cells. Positive correlations were found between the frequency of circulating Th22 cells and Th17 cells in HT (R^2 = 0.4657, p < 0.001, Fig. 1E), and also between Th22 cells and IL-22-producing Th17 cells (R^2 = 0.4312, p < 0.001; Fig. 1F). These results suggested that Th22 and Th17 subsets may have a synergetic role in HT.

Levels of IL-6 and IL-22 were increased in HT patients

Consistent with the results regarding circulating Th22 cells, serum IL-22 levels correlated with circulating Th22 cells and were significantly higher in patients with HT compared with those of the other groups (Fig. 1G, 1H). IL-6, a major Th22 cell differentiation effector [17, 19], was also higher in the sera of patients with HT compared with those of eGD and control groups (p = 0.012, p = 0.024, Fig. 1I). Furthermore, serum levels of IL-6 moderately also correlated with circulating Th22 cells in patients with HT (R^2 = 0.1793, p = 0.011; Fig. 1J).

We also measured production of IL-6 in PBMCs from some subjects in HT, uGD, and control groups. Consistent with the results in serum, PBMCs from HT patients produced significantly higher levels of IL-6 in vitro without addition of Th22 cell differentiation effector, compared with those in control (p = 0.02, Fig. 2C).

Lymphocytes from HT patients showed higher rate of differentiation into Th22 cells in vitro

Consistent with the results in serum, PBMCs from HT patients produced significantly higher levels of IL-6 in vitro without addition of Th22 cell differentiation effector, compared with those in control (p = 0.02, Fig. 2C).
Th22 cells in Hashimoto’s thyroiditis

Percentage of Th22 cells was also increased in mice of the AIT group (data not shown). We measured the secretion of IL-6 and IL-22 in splenocytes derived from some of each group. Consistent with the previous results, splenocytes from mice of the AIT group produced significantly higher levels of IL-6 and IL-22 in vitro, compared with those of the control group (p = 0.0274, p = 0.0304, Fig. 5D, 5E). These results are similar to those of the patients with HT, suggesting an enhanced differentiation of Th22 cells in AIT.

Increased numbers of Th22 cells and enhanced expression of IL-6 in splenocytes of AIT mice

The percentage of Th22 cells in CD4+ T cells was significantly higher in mice of the AIT group (4.68 ± 0.37%) than in the control group (3.45% ± 0.34%, p = 0.021, Fig. 5B, 5C). Consistent with previous reports [14], percentage of Th17 cells was also increased in mice of the AIT group (data not shown).

We measured the secretion of IL-6 and IL-22 in splenocytes derived from some of each group. Consistent with the previous results, splenocytes from mice of the AIT group produced significantly higher levels of IL-6 and IL-22 in vitro, compared with those of the control group (p = 0.0274, p = 0.0304, Fig. 5D, 5E). These results are similar to those of the patients with HT, suggesting an enhanced differentiation of Th22 cells in AIT.
Fig. 2  Differentiation of lymphocytes into Th22 cells in vitro was shown. PBMCs were cultured with or without anti-CD3, anti-CD28, anti-IFN-γ, anti-IL-4 antibodies, IL-2, IL-6, or TNF-α for 6 days. CD3⁺CD8⁻ cells represented CD4 T cells. (A) FACS gating used in the analysis for CD3⁺CD8⁺IL-22⁺ cells. (B) Representative graphs showed CD3⁺CD8⁺IL-22⁺ cells gated in A. (C) After culture, PBMCs from HT patients produced larger amounts of IL-6 in vitro. (D) After addition of IL-6 and TNF-α, PBMCs from HT patients differentiated into more polarized IL-22-producing CD4 T cells in vitro, compared with other groups. (E) In the presence of IL-6 and TNF-α, PBMCs from HT patients produced larger amounts of IL-22 in vitro, compared with the other groups. * p < 0.05. HT, Hashimoto's thyroiditis; uGD, untreated Graves' disease; eGD, euthyroid Graves' disease

Fig. 3  The correlations between percentage of Th22 cells and clinical data were shown. (A) A moderately positive correlation between percentage of Th22 cells and serum TPOAb titers was found. (B) The proportion of Th22 cells in HT patients with TPOAb titers > 2000 IU/mL was higher than those of patients with TPOAb titers < 2000 IU/mL (p = 0.0087). (C) There was no correlation between percentage of Th22 cells and levels of TSH in serum. * p < 0.05. HT, Hashimoto's thyroiditis
Th22 cells in Hashimoto’s thyroiditis

In the present study, we found distinctly more circulating Th22 cell differentiation, as well as elevated serum IL-22 levels, in patients with HT. However, this was not true of patients with GD. These results support a recent study regarding Th22 cells in onset HT [28], but they are inconsistent with another which reported elevated Th22 cells in onset GD [27]. In our work, both uGD and eGD patients were enrolled. We

**Discussion**

In this work, we investigated differentiation of circulating Th22 cells as well as related cytokines IL-6 and IL-22 in AITD patients, established polarization of Th22 cells in PBMCs from subjects in vitro. Furthermore, the correlation of circulating Th22 cells and TPOAb titers was analyzed. Finally, for the first time, we further explored Th22 cells involved in iodine-induced AIT model in NOD.H-2\textsuperscript{b4} mice.

Fig. 4 IL-22 positive lymphocytes localization in thyroid glands, histopathology of thyroids, thyroiditis scores, and serum TGAb titers in mice were shown. Immunofluorescence method was used to detect IL-22+ lymphocytes in thyroid. (A) Representative histology of thyroid glands in mice was shown with H&E stain 200×. (B) Thyroid gland sections from mice were stained for IL-22. Representative immunofluorescence images for IL-22 (red) and nucleus (blue) in thyroid tissue sections were shown. (C, D) Thyroiditis scores and serum TGAb titers in AIT group were both higher than that in control group. (E) Percentage of IL-22 positive lymphocytes was higher in thyroid glands of AIT mice than those in control mice. ** p < 0.001. AIT, autoimmune thyroiditis
failed to find any differences in circulating Th22 cells or serum IL-22 among the GD patients and healthy individuals. Our results are consistent with the findings of Figueroa-Vega et al. [11], including elevated circulating CD4⁺IL-22⁺ T cells and enhanced expression of IL-22 in thyroid glands from HT patients but not GD patients. However, they presumably viewed CD4⁺IL-22⁺ T subsets as Th17 cells, without measuring Th22 subsets.

In accordance with previous reports [24, 25], in our HT patients the percentage of Th17 cells positively correlated with numbers of Th22 cells. These results suggest that Th22 and Th17 subsets have a synergistic role in HT. The positive correlation may be due to IL-6-mediated differentiation of Th17 and Th22 cells, as shown in the in vitro experiment and as previously reported [17, 31, 32].

We showed elevated levels of IL-6, a major Th22 cell differentiation effector, were correlated with percentage of Th22 cells in HT patients. This is good evidence which supports elevated circulating Th22 cells in HT patients. However, the mechanism of elevated IL-6 in HT needs further investigation. The enhanced capability of freshly isolated PBMCs from HT patients to differentiate into Th22 cells in vitro (unlike those of GD patients) is of interest. This phenomenon could also be due to enhanced expression of IL-6 in HT, as we shown in this study. All these results are consistent and support a role for Th22 cells in the pathogenesis of HT.

In our study, we found a moderately positive correlation between Th22 cells percentage and serum TPOAb titers. This indicates that Th22 cells may be related to disease activity in HT patients. For the limitation of our study, we weren’t able to get the thyroid tissues of patients, the inflammation in which was more represen-
Th22 cells in Hashimoto’s thyroiditis

NOD.H-2<sup>h4</sup> mice are prone to spontaneous AIT; they provide a prototype murine model of HT in humans [2]. Previous studies have shown that Th17 cells have a key, independent role in thyroiditis in NOD.H-2<sup>h4</sup> mice [14]. To the best of our knowledge, this is the first report of Th22 cells in NOD.H-2<sup>h4</sup> mice. We observed increased numbers of Th22 cells in splenocytes from mice in the AIT group, accompanied by more severe thyroiditis. This may be related to the higher production of IL-6, as we showed in AIT group, which may promote the differentiation of Th22 cells. These results are highly consistent with what we found in HT patients, and prove the enhanced differentiation of Th22 cells in autoimmune thyroiditis. In addition, we found increased numbers of IL-22 positive lymphocytes infiltrated in the thyroid glands of mice in the AIT group. These indicate that Th22 cells participate in local inflammation in thyroiditis, and further suggest that Th22 cells have an important role in the pathogenesis of HT.

In summary, our results show the enhanced differentiation of Th22 cells in HT patients. For the first time, we also demonstrate Th22 cells play a role in AIT model in NOD.H-2<sup>h4</sup> mice. These suggest that Th22 cells may contribute to pathogenesis of HT. The limitation of this research is the small sample size. Further study with larger sample size needs to be conducted. And the precise role of Th22 cells in HT needs further investigation.

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Disclosure

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

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