Increased IL-35 in the circulation of patients with Graves’ disease

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Increased IL-35 in the circulation of patients with Graves’ disease

Running title: Increased IL-35 in GD

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Abstract

Introduction: The aim of this study was to explore the potential role of IL-35 in Graves’ disease (GD).

Material and methods: A total of 142 GD patients including 80 newly onset patients, 52 refractory patients and 10 remission patients and 70 normal controls (NCs) were recruited. The messenger RNA (mRNA) expressions of P35 and Epstein-Barr-virus-induced gene 3 (Ebi3) were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Serum level of IL-35 was measured by enzyme-linked immunosorbent assay (ELISA).

Results: The expression of IL-35 mRNA in new onset GD and refractory GD were both significantly higher than NC. Comparison between remission GD and NC showed no significant difference (P > 0.05). A significant increase of Ebi3mRNA expression was
observed in new onset GD compared with remission GD \((P = 0.030)\). The new onset GD showed a tendency for increased expression of serum IL-35 but without significant difference. No correlation between IL-35 expression and clinic parameters was found.

**Conclusions:** Our preliminary observations indicate that IL-35 and CD4\(^+\)P35\(^+\)Ebi3\(^+\)T cells may be involved in the pathogenesis of GD.

**Key words:** interleukin 35; CD4\(^+\)P35\(^+\)Ebi3\(^+\)T cell; Graves’ disease; immunology

**Introduction**

Graves’ disease (GD) is classified as an organ-specific autoimmune disease and is also the most common cause of clinical hyperthyroidism. The classical triad of GD is thyrotoxicosis, goitre, and ophthalmopathy. Although its exact aetiology is still unknown, GD is generally believed to be caused by immune dysfunction, especially cellular immune dysfunction. Unbalanced CD4\(^+\)T lymphocytes (CD4\(^+\)T) and relevant cytokines are major contributors to its immunological pathogenesis. These include Th1 [1, 2], Th2 [1, 3], Th17 [3–5] regulatory T cell (Treg) [4, 5], Th22 [6], and follicular helper T cell (Tfh) [7, 8] and their corresponding cytokine profile interferon-\(\gamma\) [IFN-\(\gamma\)], interleukin-4 [IL-4], interleukin-17 [IL-17], interleukin-10 [IL-10], transforming growth factor-\(\beta\) [TGF-\(\beta\)], interleukin-22 [IL-22], interleukin-21[IL-21], etc.

Interleukin-35, a new member of the IL-12 family, is a heterodimer formed by Epstein-Barr-virus-induced gene 3 (Ebi3) and P35 [9]. Human thymus-derived CD4\(^+\)Treg (nTreg) can secret IL-35 inconsecutively [10]. Whereas, iTreg35, a newly defined induced Treg (iTreg) can produce IL-35 consecutively [10]. Furthermore, a recent study found that regulatory B cell (Breg) can also secret IL-35 as well as IL-10 [11–13]. The receptor of IL-35 is a unique IL-12R\(\beta\)2:gp130 heterodimer or IL-12R\(\beta\)2:IL-12R\(\beta\)2, gp130:gp130 homodimers. IL-35R signalling is mainly through transcription factors STAT1 and STAT4, which can bind to the distinct sites of P35 and Ebi3 promoters [14]. However, a study also found that the binding of IL-35 to an IL-12R\(\beta\)2:WSX-1 heterodimer can induce the activation of STAT1 and STAT3 in Breg [14].

*In vivo*, IL-35 can suppress immune response through the suppression of Th17 proliferation, IL-17 secretion, and expansion of Treg [16]. The immunosuppression
function of IL-35 and iTr35 has been confirmed in multiple autoimmune diseases including Hashimoto’s thyroiditis (HT) [17], systemic lupus erythematosus (SLE) [18], inflammatory bowel disease (IBD) [19], and multiple sclerosis (MS) [20] and in variable animal models such as experimental autoimmune encephalomyelitis (EAE) [10] and collagen-induced arthritis (CIA) [21]. Despite these studies, the expression of IL-35 or IL-35-producing cells in GD is still not well understood. Therefore, the present study was initiated to analyse the expression of IL-35 and IL-35-producing cells in peripheral blood of GD patients and their correlation with clinical features.

Material and methods

Study subjects

A total of 142 GD patients including 80 newly onset patients, 52 refractory patients, and 10 remission patients were recruited randomly from the inpatient and outpatient department of endocrinology of Jinshan Hospital of Fudan University. Seventy normal controls (NCs) were recruited from the health check-up department of the same hospital. GD was defined by: 1) manifestations of hyperthyroidism, such as weight loss despite a hearty appetite, fatigue, irritability, anxiety, tremor, palpitations, and so on; 2) biochemical confirmation of hyperthyroidism including decreased serum thyrotropin-stimulating hormone (TSH), increased free triiodothyronine (FT3) and/or free tetraiodothyronine (FT4), the positive circulation of thyrotropin receptor antibody (TRAb), or thyroid peroxidase antibodies (TPOAb) (normal range: TSH 0.27–4.2 mU/l, FT4 12–22 pmol/L, FT3 3.1–6.8 pmol/L, TRAb 0–1.75 IU/L, TPOAb 0–34 IU/ml); 3) the presence of diffused enlarged thyroid gland; 4) the presence of thyroid-associated ophthalmopathy (TAO); and 5) the presence of dermopathy, which is often shown as pretibial myxoedema. The demographic and clinical characteristics, including thyroid function parameters (FT3, FT4, TSH) and TRAb of each subject, were gathered.

Refractory GD was defined as those the discontinue antithyroid drugs (ATD) should be persistent for more than two years after initiation of the therapy and were still positive for TRAb [22]. Remission GD was defined as those who maintained a euthyroid status for more than one year after withdrawal of ATD [22].

Sample processing
Blood samples of each participant were collected into Vacutainer tubes containing ethylenediamine tetra-acetic acid (EDTA). PBMCs were isolated by Ficoll-Hypaque gradient and washed with phosphate-buffered saline (PBS) (Hyclone, USA). PBMCs were then resuspended with TRIzol reagent (Invitrogen, Bleiswijk, the Netherlands) to extract mRNA for quantitative real-time PCR (qRT-PCR). Fasting blood samples were clotted for 30 min at room temperature and then centrifuged at 3000 g for 10 min to collect serum specimens. All samples were stored at –80°C.

**mRNA isolation and quantitative real-time PCR**

Total mRNAs were isolated from PBMCs with TRIzol reagent. The quantity and purity of mRNAs were measured by the absorbance on an Epoch Multi-Volume Spectrophotometer System (Biotek, USA) at 260 nm and 280 nm. MRNA samples with A260/A280 between 1.8 and 2.0 were then reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa, Japan) following the commercially available protocol. The cDNA samples were stored at -20°C for next analysis. As for qRT-PCR, the ABI PRISM 7300 Fast Real-Time PCR system (BIO-RAD) with SYBR Premix Ex TaqTM II (Perfect Real Time) (TaKaRa, Japan) were used according with the manufacturers’ guidelines. Gene expressions were normalised by β-actin. The primer sequences of P35, Ebi3, and β-actin are listed in Table I. The amplification and melting curves were checked after reaction.

**Enzyme-linked immunosorbent assay**

Serum IL-35 levels were measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Biolegend, USA) in strict accordance with the manufacturers’ protocols.

**Statistical analysis**

All statistical analyses were conducted using SPSS 17.0. Data were expressed as the mean ± SD. Balance between groups was evaluated by the continuity correction in Chi-square test. For the unbalanced groups, covariance analysis was used. Differences between groups were compared using Mann-Whitney-U-test. Spearman correlation test was used to assess the associations between IL-35 levels and clinical parameters. P < 0.05 was considered as statistically significant.
Results

The expression of P35mRNA and Ebi3mRNA were increased in GD patients

Thirty new onset GD, 22 refractory GD, 10 remission GD, and 30 age- and gender-matched NC were enrolled for qRT-PCR. The demographic and clinical data of the participants are shown in Table II. Compared to the other three groups, the age of remission GD patients was relatively high and lacked balance between groups. Hence, the comparisons between remission GD and other groups were conducted using covariance analysis. As shown in Figure 1, the expression of P35mRNA and Ebi3mRNA in new onset GD and refractory GD were significantly higher than in NC. Comparison between remission GD and NC showed no significant difference (P > 0.05). A significant increase of Ebi3mRNA expression was observed in new onset GD compared with remission GD (P = 0.030). The expression of P35mRNA tended to be higher in new onset GD than remission GD but without significant difference (P > 0.05). Unfortunately, spearman correlation analysis revealed no correlation between clinical parameters (FT3, FT4, TSH, and TRAb) and any kind of mRNA expression (P > 0.05).

The new onset GD showed a tendency for increased expression of serum IL-35

Serum from 30 new onset GD, 30 refractory GD, and 20 age- and gender-matched NC were collected for ELISA. However, the positive rate of IL-35 in total samples was just approximately 30%. Demographic and clinical data of those subjects are shown in Table III. Serum IL-35 concentration among new onset GD (8.43 ± 5.88 ng/ml), refractory GD (7.40 ± 4.66 ng/ml) and NC (5.75 ± 4.27 ng/ml) had no statistical difference (P > 0.05), but there was an increasing tendency in new onset GD compared with NC. No correlation was found between IL-35 concentration and clinical parameters (P > 0.05).

Discussion

Although the fundamental pathogenesis of GD remains unknown, it is generally believed to be a multifactorial disease caused by environmental factors and genetic susceptibility. The interactions between them lead to the initiation of immune disequilibrium including cell-mediated and humoral immune dysfunctions. As for cell-mediated immune dysfunction, CD4+T cell subsets are mainly studied cells. Among
them, induced Treg (iTreg) cells are divided into Th3 (TGF-β-induced-iTreg), Tr1 (IL-10-induced-iTreg), and iTr35 (IL-35-induced-iTreg), according to their generation mechanisms [23]. iTr35 is a relatively newly defined iTreg subset, which has a highly restricted CD4+/Foxp3+/Ebi3+/P35+/IL10−/TGF-β− genetic signature [10]. iTr35 can suppress immune response through the suppression of Th17 proliferation and expansion of Treg through secretion of IL-35 [10]. Th17/Treg cell imbalance is a well-known cell-mediated immune dysfunction mechanism of GD [4, 24], which raises the possibility that IL-35 may play a partial role in its pathogenesis.

In the present study, inter-group comparison showed that the P35 and Ebi3 mRNA expression levels of new onset and refractory GD were both significantly higher than controls. While there was no difference between remission GD and controls. These data suggest that IL-35 might participate in the pathogenesis of GD. IL-35 is well documented as an anti-inflammatory cytokine in multiple autoimmune diseases; however, as its subunit, P35 is found to be a pro-inflammatory factor [25] or just act as a ligand [26]. Characterisation of Ebi3 reveals that it has a dual role, having either pro-inflammatory or anti-inflammatory properties [27]. Therefore, it is Ebi3 that may mainly exert the immunological function of IL-35 [16]. The Ebi3mRNA expression, but not P35mRNA, in new onset GD was significantly higher than remission GD in our subsequent intra-group comparison. This might further verify the hypothesis above and suggest that the Ebi3mRNA expression is related to the activity of GD and could be downregulated along with the remission of disease. Levels of IL-35 in approximately 70% of the participants were undetectable due to its low density in serum. This may be the reason why the IL-35 serum level was not fully consistent with its mRNA expression. Also, we could not exclude the possibility of post-transcriptional regulation of IL-35.

Variable studies have confirmed the immunoregulatory role of IL-35 in many other autoimmune diseases. In active SLE, serum IL-35 levels were significantly decreased and negatively correlated with the SLE disease activity parameters (SLEDAI) [18]. Similar results were found in IBD: serum IL-35 levels were significantly decreased in IBD patients and correlated inversely with UC activity [19]. In MS, the serum levels of IL-35 in treated patients were significantly higher than those in the controls, but no significant difference between untreated MS patients and controls was found [20]. Serum IL-35 levels were lower in the HT group when compared with the subclinical
HT group and controls and inversely associated with clinic parameters [17]. However, our study revealed opposite results: upregulation of IL-35 in GD. As we know, IL-35 is an anti-inflammatory cytokine; thus, we speculated that its elevation might reflect a compensational response or protective mechanism. Besides, the immunopathogenesis of GD contains a complicated and multifactorial regulatory network; a single immunocyte or cytokine could not possibly explain its mechanism completely.

**Conclusions**

Our preliminary observations indicate for the first time that IL-35 may be involved in the pathogenesis of GD. Other studies of IL-35 expression and its role are mandatory for further elucidation of the pathogenesis of Graves’ disease.

**Acknowledgments**

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**Availability of data and materials**

Data are available on request to the authors.

**Authors’ contributions**

Research concept and design: Jin-an Zhang and Shuai Meng; collection and/or assembly of data: Shuai Meng, Jin-an Zhang, Jian Xu, and Shuangtao He; data analysis and interpretation: Shuai Meng, Ni Yan, Jian Xu; writing the article: Shuai Meng; critical revision of the article: Jin-an Zhang; final approval of the article: Jin-an Zhang, Shuai Meng, Ni Yan, Jian Xu, and Shuangtao He.

**Competing interest**
The authors declare that they have no competing interests.

**Ethical approval and consent to participate**

The research protocol was approved by the Ethics Committee at the Jinshan Hospital of Fudan University (2014-04). Written, informed consent was obtained from each study participant.

**References**


Table I. Sequences for qRT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Product (bp)</th>
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<tbody>
<tr>
<td>P35</td>
<td>Forward:5’-TCCTCCCTTGAAACCGGA-3’</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Reverse:5’-TGACAACGTTTGGAGGAC-3’</td>
<td></td>
</tr>
<tr>
<td>Ebi3</td>
<td>Forward:5’-AGCCACGTCTTCATCCTC-3’</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>Reverse:5’-TACTTGCCCAGGCTATTG-3’</td>
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</tr>
<tr>
<td>β-actin</td>
<td>Forward:5’-CATTGCCGACAGGATGCAG-3’</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>Reverse:5’-CTCGTCATACTCCTGCTTG-3’</td>
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Table II. Demographic and clinical data of participants — participants for qRT-PCR

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>New onset GD</th>
<th>Refractory GD</th>
<th>Remission GD</th>
<th>NC</th>
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<tbody>
<tr>
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<td>Male</td>
<td>Female</td>
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<td></td>
<td>6</td>
<td>24</td>
<td>3</td>
<td>19</td>
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<tr>
<td>Age [years]</td>
<td>33.8 ± 10.2</td>
<td>34.1 ± 11.8</td>
<td>50.9 ± 7.52</td>
<td>33.0 ± 9.91</td>
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<td>Thyroid function</td>
<td>FT3 [pmol/L]</td>
<td>30.4 ± 12.0</td>
<td>6.68 ± 4.44</td>
<td>4.52 ± 0.189</td>
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<td></td>
<td>FT4 [pmol/L]</td>
<td>62.5 ± 29.0</td>
<td>21.9 ± 17.5</td>
<td>16.4 ± 2.68</td>
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<tr>
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<td>TSH [mIU/L]</td>
<td>0.0061 ± 0.0032</td>
<td>1.11 ± 0.930</td>
<td>2.18 ± 1.06</td>
</tr>
</tbody>
</table>
Table I. Demographic and clinical data of participants — participants for ELISA

<table>
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<tr>
<th>Characteristics</th>
<th>New onset GD</th>
<th>Refractory GD</th>
<th>NC</th>
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</thead>
<tbody>
<tr>
<td>Gender</td>
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<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Age [years]</td>
<td>38.8 ± 15.3</td>
<td>40.3 ± 14.2</td>
<td>31.0 ± 8.50</td>
</tr>
<tr>
<td>Thyroid function</td>
<td></td>
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<tr>
<td>FT3 [pmol/L]</td>
<td>24.0 ± 14.0</td>
<td>7.26 ± 4.62</td>
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<tr>
<td>FT4 [pmol/L]</td>
<td>57.3 ± 28.4</td>
<td>17.6 ± 6.90</td>
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<tr>
<td>TSH [mIU/L]</td>
<td>0.00563 ± 0.00177</td>
<td>0.906 ± 0.873</td>
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<tr>
<td>TRAb [IU/L]</td>
<td>16.8 ± 9.05</td>
<td>13.9 ± 12.8</td>
<td></td>
</tr>
<tr>
<td>IL-35 [ng/ml]</td>
<td>8.43 ± 5.88</td>
<td>7.40 ± 4.66</td>
<td>5.75 ± 4.27</td>
</tr>
</tbody>
</table>

mRNA: messenger RNA; NCs: normal controls; Ebi3: Epstein-Barr-virus-induced gene 3; Treg: regulatory T cell; Th: follicular helper T cell; IFN-γ: interferon-γ; IL-4: interleukin-4; IL-17: interleukin-17; IL-10: interleukin-10; TGF-β: transforming growth factor-β; Breg: regulatory B cell; HT: Hashimoto’s thyroiditis; SLE: systemic lupus erythematosus; IBD: Inflammatory bowel disease; MS: multiple sclerosis; EAE: experimental autoimmune encephalomyelitis; CIA: collagen-induced arthritis; TSH: thyrotropic-stimulating hormone; TPOAb: thyroid peroxidase antibodies; TAO: thyroid-associated ophthalmopathy; ATD: antithyroid drugs; EDTA: ethylenediaminetetra-acetic acid; DMSO: dimethyl sulfoxide; PBS: phosphate-buffered saline; iTr35: IL-35-induced-iTreg; SLEDAI: SLE disease activity parameters.
ELISA — enzyme linked immunosorbent assay; GD — Graves’ disease; NC — normal control; FT3 — free triiodothyronine; FT4 — free tetraiodothyronine; thyrotropic-stimulating hormone; TRAb — thyrotropin receptor antibody; IL-35 — interleukin 35

**Figure 1.** P35mRNA and Ebi3mRNA expression in peripheral blood mononuclear cells (PBMCs). The expression of P35mRNA and Ebi3mRNA in new onset Graves’ disease (GD) and refractory GD were both higher than normal control (NC) [P35: new onset GD vs. NC (*P = 0.028), refractory GD vs. NC (**P = 0.031); Ebi3: newly onset GD vs. NC (#P = 0.039), refractory GD vs. NC (##P = 0.042)]. There was no difference in both P35 mRNA and Ebi3mRNA expression between remission GD and NC. Intra-group comparison found Ebi3mRNA expression was more elevated in new onset GD than in remission GD (###P = 0.030)

**Figure 2.** Serum IL-35 concentration among new onset Graves’ disease (GD),
refractory GD, and normal control (NC) have no statistical difference