Selenium upregulates CD4^{+}CD25^{+} regulatory T cells in iodine-induced autoimmune thyroiditis model of NOD.H-2^{h4} mice

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Abstract. Selenium (Se) is required for thyroid hormone synthesis and metabolism. Se treatment reduces serum thyroid-specific antibody titers in patients with autoimmune thyroiditis (AIT), but the exact mechanism is not clear. We investigated the effects of Se treatment on CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells (Treg) in a iodine-induced autoimmune thyroiditis model. NOD.H-2^{h4} mice were randomly divided into control, AIT untreated, and AIT with Se treatment groups. Mice were fed with 0.005% sodium iodine (NaI) water for 8 weeks to induce AIT. Se-treated mice received 0.3 mg/L sodium selenite in drinking water. The AIT mice had fewer Treg cells and reduced Foxp3 mRNA expression in splenocytes compared with the controls (p < 0.01). The percentage of Treg cells and expression of Foxp3 mRNA were increased by Se treatment (as compared with untreated AIT mice, p < 0.05). Mice that received Se supplementation also had lower serum thyroglobulin antibody (TgAb) titers and reduced lymphocytic infiltration in thyroids than untreated AIT mice. These data suggest that CD4^{+}CD25^{+} T cells play an important role in the development of AIT. Se supplementation may restore normal levels of CD4^{+}CD25^{+} T cells by upregulating the expression of Foxp3 mRNA in mice with AIT.

Key words: Selenium, NOD.H-2^{h4} mice, Autoimmune thyroiditis, Regulatory T cells

SELENIUM (Se) is an essential trace element that, like iodine, influences a number of endocrine processes, most notably, those involved in thyroid hormone synthesis, activation, and metabolism [1]. Furthermore, Se has an important impact on immune function. Se deficiency is accompanied by a loss of immune competence; both cell-mediated and humoral immunity may be impaired [2]. Recently, several clinical studies have demonstrated that in patients with autoimmune thyroiditis (AIT), Se supplementation reduced the titers of thyroid peroxidase antibody (TPOAb) significantly as compared to the control subjects receiving placebo, suggesting that Se supplementation might be an effective treatment for AIT [3-7].

NOD.H-2^{h4} mice, which express I-A^{k}, H-2K^{k}, and D^{d} on the NOD background, develop autoimmune thyroiditis when given iodide in drinking water. It is a prototype murine model of Hashimoto’s thyroiditis (HT) in humans [8]. HT is one of the most prevalent autoimmune thyroid diseases and affects more than 10% of females and 2% of males with thyroid disease. HT is mediated by CD4^{+} T cells and characterized by lymphocytic infiltration into thyroid and by thyroid-specific antibody in serum. CD4^{+}CD25^{+} regulatory T lymphocytes (Treg cells) contribute to the maintenance of peripheral self-tolerance and the prevention of autoimmunity in animals and humans. The development of Treg cells has been shown to be programmed by the transcription factor Foxp3 [9]. Several studies have reported that experimental mice develop multi-organ autoimmune diseases and inflammations, including thyroiditis, after injection of anti-CD25 antibody, resection of the thymus, or knock out of Foxp3. This suggests that development of many autoimmune diseases could be prevented or sup-
pressed by therapy that increased numbers of Treg cells or enhanced the function of Treg cells [10].

Although the immunological benefit of Se treatment has been demonstrated in AIT patients, the mechanism of the effect has not been elucidated. The present study was designed to investigate whether Se treatment affected CD4\(^+\)CD25\(^+\) regulatory T cells in NOD.H-2\(^b\) mice with AIT.

**Materials and Methods**

**Mice**

NOD.H-2\(^b\) mice, purchased from Jackson Laboratory (Bar Harbor, ME, USA), were bred and raised under specific pathogen free conditions in 12 h light/12 h dark cycles in the animal facility of China Medical University. All animal care and experimental procedures were performed according to the Guideline for Animal Experimentation with the approval of the Animal Ethics Committee of China Medical University. The mean contents of iodine and selenium in the animal feed were 460 µg/kg and 100 µg/kg, respectively.

The mice were used in experiments at 4 weeks of age and had a mean weight of 20 g. The 150 male NOD.H-2\(^b\) mice were randomly divided into three groups: a control group was given sterile water, a AIT group given 0.005% (50 mg/L) sodium iodine (NaI) in the drinking water (100 times over the normal iodine intake) during the study period, and a AIT/Se supplementation group given NaI for 8 weeks and then 0.3 mg/L sodium selenite (3 times normal Se intake) in drinking water for 8 weeks. Animals were anesthetized and sacrificed at week 8 or week 16 of the experiment.

**Assessment of autoimmune thyroiditis**

At the experimental end points (8 or 16 weeks), mice were weighed and anesthetized by an intraperitoneal injection of 10% chloral hydrate. Thyroid tissues were removed and then washed with cold normal saline, dried on a pad of filter paper, and weighed on an electronic balance (BS210S, Sartorius, Germany). One thyroid lobe from each mouse was fixed in 10% paraformaldehyde at least 24 h and embedded in paraffin. Sections of 5-µm thickness were prepared and stained with hematoxylin and eosin (HE). Histological changes in the thyroid tissue were observed under light microscopy (BX51/BX52, Olympus, Japan). Extent of lymphocytic infiltration was assessed as previously described [11]. Briefly, HE-stained thyroid sections were graded on the following scale according to the approximate area of lymphocytic infiltration: 0 = normal; 1+ = 1–10%; 2+ = 10–30%; 3+ = 30–50%; 4+ >50%. The thyroiditis scores were expressed as means of at least three non-contiguous sections from each thyroid gland.

**Serum thyroglobulin antibody (TgAb) measurements by ELISA**

Blood was collected from the heart, incubated at room temperature at least 2 h and serum was separated by centrifugation at 3000 rpm for 20 min and stored at -70°C until use.

Thyroid gland tissues were homogenized and centrifuged. Mouse Tg (MTg) was obtained from the supernatant by salting out, and then purified by repeat gel filtration on Sephadex G-20 (Pharmacia, USA). Samples were stored at -70°C until analysis [12]. Levels of TgAb were assessed in duplicate by indirect ELISA using serum from individual mice as described previously [13]. Briefly, sera were diluted to 1:100 with PBS and incubated on 96-well EIA/RIA Plates (Corning, USA) coated with 10 µg/mL MTg. Peroxidase-labeled goat anti-mouse immunoglobulin G (1:250 dilution, Sigma, USA) was used as the secondary antibody. The color change of tetramethyl benzidine (TMB) was measured at 450 nm by a microtiter plate reader (Bio-Rad 680, USA). TgAb levels were expressed as optical density (ODs) values.

**Serum selenium determination**

Serum selenium concentrations were measured in duplicate using an inductively coupled plasma mass spectrometry (ICP-MS, Perkin-Elmer SCIEX ELAN 9000, USA). Blood serum diluted 40-fold with an acidic solution consisting of nitric acid (1%), X-triton (0.1%), and 1-butanol (0.8%). A calibration curve was established for 1 to 400 µg/L (\(r^2 > 0.99\)). The limit of detection was 0.5 µg/mL. The between-run coefficient of variation (CV) was 6.59% and the within-run CV was < 2%.

**Flow cytometry**

Spleens were harvested from freshly sacrificed mice at the indicated time points and pressed through a 200-gauge stainless steel mesh. Tissue was suspended in 10 mL of PBS and centrifuged at 1000 rpm for 10 min at room temperature. Erythrocytes were removed by
addition of 3 mL lysis solution, incubation for about 3 min, and centrifugation at 1000 rpm for 10 min. The cells were resuspended in 1 mL flow cytometry staining buffer after being washed once with PBS and were then counted under an inverted light microscope.

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells were detected by multicolor flow cytometry. About 10<sup>6</sup> prepared splenic mononuclear cells were added to each tube and were stained with approximately 0.125 μg of FITC-conjugated anti-CD4 (RM4-5, eBioscience, USA) and 0.06 μg of APC-conjugated anti-CD25 (PC61.5, eBioscience, USA) antibodies, incubated at 4°C in the dark for about 30 min, and then washed in cold flow cytometry staining buffer. For Foxp3 staining, cells were further fixed and permeabilized by a commercial cell fixation/permeabilization kit (eBioscience, USA), which following the instruction of the kit. Then the cells were incubated with 0.2 μg PE-conjugated anti-mouse Foxp3 antibody (FJK16s, eBioscience, USA) or isotype control consequently. Flow cytometric analysis was performed on a FACScan flow cytometer (BD Biosciences, USA) using winMDI 2.9 software. As shown in Fig.2A, samples of splenic mononuclear cells were firstly separated by forward and side scatter, and the presumed lymphocyte population was gated (R1). Secondly, the selected lymphocytes stained with anti-CD4 were analyzed, and the CD<sup>4</sup> population of lymphocytes was gated (R2). Finally, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells were measured using anti-CD25 and anti-Foxp3 antibodies under the two Gates.

**Quantitative real-time RT-PCR analysis**

Total RNA was extracted from splenic cells with TRIzol reagent (Invitrogen, USA) following the manufacturer’s instructions. RNA integrity was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. The OD260/280 absorbance ratio was between 1.9 and 2.0 in each RNA sample. One microgram of total RNA was used to prepare cDNA. A reverse transcriptase kit (PrimeScript<sup>TM</sup> RT reagent Kit, TaKaRa) was used for complementary DNA (cDNA) synthesis (37°C for 15 min, followed by 85°C for 5 s on an ABI 9700 PCR meter). Transcripts were quantified by the Rotor-Gene 3000 real-time PCR System (Corrett Research Inc, AUS) using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Takara) as the manufacturer’s instructions. Reactions began with a 10-s hot-start activation of the Taq polymerase at 95°C, followed by 40 to 45 cycles of amplification in three steps (denaturation at 95°C for 5 s, followed by a 30-s annealing at 60°C and 30-s extension at 72°C). The reactions were carried out in a total volume of 25 μL in a 0.2-mL flat cap PCR tube (Axygen, USA). Foxp3 primers were: 5’- GCC CCT TCT CCA GGA CAG A-3’ (forward primer) and 5’- GCT GAT CAT GGC TGG GTT GT-3’ (reverse primer). GAPDH was used as an endogenous reference with primers: 5’-TGG TGA AGG TCG GTG TGA AC-3’ (forward primer) and 5’-CCA TGT AGT TGA GGT CAA TGA AGG-3’ (reverse primer). Samples were run in triplicate and the data were analyzed using Rotor-Gene Real-Time Analysis Software 6.0.

**Statistical analysis**

All results are expressed as the mean value ± standard deviation (SD). An independent-sample t test was applied for statistical analyses between two different groups using SPSS 16.0 software package (SPSS Inc, USA). A p value less than 0.05 was considered statistically significant.

**Results**

**Weight of thyroid tissues**

As shown in Table 1, the weights of thyroids in AIT group, received NaI in their drinking water for 8 weeks, were increased significantly compared to the control group (p < 0.01); After 16 weeks of iodine supplementation, the thyroid weights were increased further in AIT group. The enlarged thyroids were lighter in color and the texture was tougher than that of normal thyroids. Although the body weight of mice increased gradually with the age, we observed no significant differences in the three groups. As shown in Fig. 1A, the relative weights of thyroids in the Se treated group were decreased compared to the AIT group (p < 0.01).

**Histopathology of thyroids**

In this study, lymphocytic infiltration was observed in thyroids in about 80% of NOD.H-2<sup>4</sup> mice given 0.005% NaI in water for 8 weeks; scores of thyroiditis ranged from 1+ to 3+. Lymphocytic infiltration was more severe at 16 weeks than 8 weeks (3.00 ± 0.94 vs. 1.75 ± 0.71, p = 0.007). These results were consistent with our recent study of NOD.H-2<sup>4</sup> mice [13]. In the Se treated mice, the severity of lymphocytic infiltration in thyroids was less than that in AIT-group mice.
Serum TgAb titers were significantly elevated in the AIT group compared to the control group at 8 weeks (0.69 ± 0.24 vs. 0.46 ± 0.05, p < 0.01). At 16 weeks, TgAb titers remained high in the AIT group (0.66 ±
Fig. 2  Flow cytometric analysis of CD4^+CD25^+Foxp3^+ regulatory T cells and quantitative real-time RT-PCR measurement of Foxp3 mRNA expression.
A) An example of a flow cytometry result showing the method for the assessment of CD4^+CD25^+Foxp3^+ T cells. Splenic mononuclear cells were stained with FITC-labeled anti-mouse CD4, APC-labeled anti-mouse CD25, and PE-labeled anti-mouse Foxp3 antibodies and analyzed using FACS.
B) The percentage of CD4^+CD25^+Foxp3^+ T cells were significantly reduced in AIT-group mice compared with control mice at 8 and 16 weeks (p < 0.01, n = 10 in each group). Se treatment increased the percentage of CD4^+CD25^+Foxp3^+ T cells as compared with AIT-group mice at 16 weeks (p < 0.05, n = 10 in each group).
C) Foxp3 mRNA levels were significantly reduced in AIT-group mice (n=10) compared with control mice (n=7) at 8 and 16 weeks (p < 0.01). In Se-treated AIT mice, Foxp3 mRNA levels were significantly higher than that in AIT-group mice (p < 0.05, n = 10 in each group).

Discussion
In this study, AIT was induced in NOD.H-2^h4 mice by exposure to NaI in the drinking water as previously described [13]. The vast majority of these mice (80%) had lymphocytic infiltration in thyroids with severity scores of 1+ to 3+ and increased thyroid weight and serum TgAb titers relative to control mice. Treatment with selenium via drinking water decreased the serum TgAb titer. This result was in accordance with those observed in clinical trails. Supplementation of 200 μg Se per day suppressed anti-thyroperoxidase antibody response.
(TPOAb) levels in patients with AIT in low dietary Se intake [7], in mild selenium deficiency [3], and in a borderline Se sufficient region [4]. There was an additional decrease of TPOAb titers with the extension of Se supplementation lasted for more than 3 or 6 months [3-5, 7, 14].

Although NOD.H-2\(^h\) mice were well known for AIT model, the exact immune mechanism of this model was still unknown. In the present study, we found that NOD.H-2\(^h\) AIT mice fed with iodine had a lower percentage of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells. Treg cells maintain immunological unresponsiveness to self-antigens and suppress excessive immune responses deleterious to the host. As a specialized subpopulation of negative regulatory T cells, Tregs participate in the establishment and maintenance of peripheral self-tolerance. Deficits in the numbers and/or function of Treg cells lead to autoimmune diseases. Foxp3, a forkhead winged helix family transcriptional regulator, is a critical molecular switch for the genetic programming of Treg cell development and function. In mice, the majority of peripheral and thymic Foxp3\(^+\) cells are CD4\(^+\)CD25\(^+\) T cells and a genetic deficiency in Foxp3 results in an autoimmune pathology secondary to the loss of CD4\(^+\)CD25\(^+\) Treg cells [15, 16]. Depletion of CD4\(^+\)CD25\(^+\) Treg cells in NOD.H-2\(^h\) mice by treating with anti-CD25 antibody 4 days before the initiation of NaI supplementation increases the severity of thyroiditis and TgAb titers [17]. B cell-deficient NOD.H-2\(^h\) mice are AIT resistant and do not develop AIT even when reconstituted with B cells or given passive anti-murine Tg autoantibodies. However, when B cell-deficient NOD.H-2\(^h\) mice are first given weekly injections of anti-CD25 antibody and then given NaI in drinking water, the mice develop AIT with thyroid lesions similar to those observed in wild-type NOD.H-2\(^h\) mice with AIT [18]. Adoptive transfer of CD4\(^+\)CD25\(^+\) T cells restored resistance to experimental autoimmune thyroiditis (EAT) induction in CD25\(^+\) T cell-depleted B10 mice [19]. Recent studies also show that Treg depletion enables thyroiditis induction with mouse thyroglobulin (mTg) in traditionally-resistant mice and mTg-induced, Treg-mediated tolerance protects against EAT induction in genetically-susceptible mice [20]. The naturally-existing CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs influence the thyroiditis development in naive susceptible mice and are required for induction of antigen-specific tolerance [21]. Granulocyte-macrophage colony stimulating factor suppresses the EAT and increases numbers of CD4\(^+\)CD25\(^+\) regulatory T cells and IL-10 production [22]. TNF-related apoptosis-inducing ligand also suppresses the development of experimental autoimmune diseases, including EAT in mice, by increasing the frequency of Treg cells [23]. These results indicate that the deficiency of Tregs may play a critical role on the pathogenesis of thyroiditis in mice, and the important role of Tregs in maintaining self-tolerance.

Adequate nutritional status is essential to proper immune system function. Se has been shown to enhance the proliferation and activity of T lymphocytes and other immunocompetent cells [24-26]. In the present study, we observed that Se supplementation increased the frequency of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells and enhanced expression of Foxp3 in vivo. These changes were accompanied by suppressed TgAb titers and reduced thyroiditis. Thus the benefit of Se treatment may be due to the increase of CD4\(^+\)CD25\(^+\) regulatory T cells. Further study is required to clarify the molecular mechanism of action of Se on CD4\(^+\)CD25\(^+\) regulatory T cells.

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