

An improved method for the establishment of a model of Graves' disease in BALB/c mice

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Abstract. The present study aimed to develop a stable Graves' disease (GD) model in BALB/c mice by immunization and electroporation (EP). A total of 90 mice were divided into experimental (n=50), control (n=20) and blank (n=20) groups. The recombinant plasmid pcDNA3.1/thyroid-stimulating hormone (TSH) receptor 268 was constructed and injected into the bilateral gastrocnemius of experimental group mice at weeks 1, 4, 7 and 10. Equal volumes of saline were injected into the control and blank groups at the same time. The experimental and control groups were subjected to EP at the same time and location to enhance immunization. The levels of total serum thyroxine (T₄) and serum TSH were examined by radioimmunoassay and immunoradiometric assay, respectively. The levels of serum thyrotropin receptor N-terminal (TRAb N) and C-terminal (TRAb C) antibodies were assessed by ELISA. Whole body pertechnetate (^{99m}TcO₄⁻) imaging was performed. Mouse weight and thyroid morphology and pathology were analyzed. The GD BALB/c mouse model was successfully established, with a positive rate of 79.17% (38/48). T₄ levels increased from baseline levels of 12.05±4.23 to 52.51±23.58 ng/ml by week 12 (P<0.0001). TSH levels decreased from baseline levels of 5.53±2.78 to 1.43±0.89 μIU/ml by week 12 (P<0.0001). TRAb N antibody levels increased from baseline levels of 0.006±0.002 to 0.278±0.106 mIU/ml by week 12 (P<0.0001). TRAb C antibody levels increased from baseline levels of 11.111±2.808 to 46.701±26.436 arbitrary units/ml by week 12 (P<0.0001). At week 21, TSH levels remained reduced compared with pre-immunization levels (P<0.0001). Although T₄, and TRAb N and C levels decreased, they remained increased compared with preimmunization levels (P<0.0001, P<0.0001, P=0.001). There were no significant alterations in antibody

levels between the control and blank groups. Following four immunizations, the uptake of ^{99m}TcO₄⁻ by the thyroid was significantly increased in the experimental group. The mean weight of the experimental mice was significantly reduced compared with the control and blank groups (all P<0.0001). Furthermore, the thyroid glands of the immunized mice were enlarged and exhibited lymphocyte infiltration, fewer colloid nodules and an increased height of epithelial cells. In conclusion, by injecting recombinant plasmid pcDNA3.1/TSHR268 and EP, a GD mouse model was successfully established.

Introduction

Thyrotoxicosis is a condition with multiple etiologies, manifestations and potential therapies. The term 'thyrotoxicosis' refers to a clinical state that results from increased thyroid hormone levels in tissues. The most common manifestation is termed Graves' disease (GD). In China, the prevalence of GD is approximately 1.1-1.4% (1). GD is an organ-specific autoimmune thyroid disease with a genetic predisposition. Typical features of GD include high metabolic syndrome, goiter and Graves' orbitopathy (GO). Patients with GD should be treated with any of the following modalities: Anti Thyroid Drugs (ATDs), radioactive iodine (RAI) therapy, or thyroidectomy. In China, ATD therapy has been the most preferred by physicians, however recent developments have suggested an increase in the use of RAI therapy to reduce the use of ATDs. In GD, the generation of an autoantibody, thyrotropin receptor antibody (TRAb), against the thyroid-stimulating hormone (TSH) receptor (TSHR) causes continuous stimulation of the thyroid gland and hyperthyroidism (2,3). The precise immunological mechanism of TRAb production, however, remains elusive. Previous studies have indicated that TRAb is detected in the serum of most patients with GD (4,5). TRAb is an important independent risk factor for GD (6). The titer of TRAb influences the duration of ATD therapy and the dose of RAI therapy (7). The establishment of a GD animal model allows the study of its etiology and potential therapeutic targets. In recent years, various animal models of GD have been developed that have demonstrated an autoimmune response to TSHR. Two attempts were made to immunize mice using human TSHR: one via the injection of TSHR-transfected cells simultaneously expressing MHC class II (8), and the other by genetic immunization using TSHR cDNA (9). These immunized mice demonstrated typical phenotypes associated

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with hyperthyroidism, however the response was temporary and TRAb was produced against non-self TSHR. The process of autoantibody production in these mice presumably lacks the persistent abrogation of self-tolerance. The present study investigated an improved method for inducing GD in BALB/c mice by injecting the recombinant plasmid pcDNA3.1/TSHR268 and performing electroporation (EP). This novel method has numerous advantages, including a short model cycle, high success rates, stability and reproducibility. This model may not precisely mimic human disease; however, the present study forms a theoretical basis for further study on the etiology and pathogenesis of GD, and for the development of novel therapeutic strategies.

Materials and methods

Materials. A total of 90 female BALB/c mice (age, 6-8 weeks; weight, 18-20 g) were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). All the animals were housed in individual cages with a constant temperature (18-20°C) and humidity (65-69%) at 12/12 h light/dark cycle with free access to food and water. The experimental procedures were approved by the animal ethics committee of Tianjin Medical University. EP was performed using an ECM[®]830 electroporator (BTX; Harvard Apparatus, Holliston, MA, USA). Pertechnetate (^{99m}TcO₄⁻) was produced by a molybdenum technetium generator (China Institute of Atomic Energy, Beijing, China). The mice were imaged using a single-photon emission computer tomography (SPECT) machine (Discovery NM-670; GE Healthcare Life Sciences, Shanghai, China).

Preparation of recombinant plasmid pcDNA3.1/TSHR268. Total RNA was extracted from healthy human thyroid tissue and reverse transcribed. The tissue was obtained from male and female adult patients, during the resection of benign thyroid nodules. The Total RNA extraction kit (Protein and RNA Extraction Kit) and reverse transcription kit (PrimeScript 1st Strand cDNA Synthesis kit) were supplied by Takara Biotechnology Co., Ltd. (Dalian, China). The experiments were performed following the manufacturer's protocol. The experimental procedures and materials were approved by the ethics committee of Tianjin Medical University General Hospital. Human TSHR 164-967 bp (TSHR268) was amplified by polymerase chain reaction (PCR) using the following primer sequences: Forward, 5'-ACGCGAATTCGCCACCATGGGGTGTTCGTCTCC-3 and reverse, 5'-AGGCGGATCCCTGATTCTTAAAAGCACAGCAGT-3. The PCR reaction mixture contained: 0.25 μl Pyrobest[™] DNA polymerase (Takara Biotechnology Co., Ltd.), 5 μl 10X Pyrobest buffer, 4 μl 2'-deoxynucleoside 5'-triphosphates mix (2.5 mmol/l), 1 μl upstream primer (20 μmol/l), 1 μl downstream primer (20 μmol/l), 2.5 ng template cDNA and deionized water to a final volume of 50 μl. The cycling conditions were as follows: Pre-denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 30 sec and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR product was cloned into the pcDNA3.1 vector by pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA,

USA). The experimental procedure was conducted according to the manufacturer's protocol.

Experimental groups. The BALB/c mice were randomly divided into experimental (n=50), control (n=20) and blank (n=20) groups. Mice were injected with the recombinant plasmid pcDNA3.1/TSHR268 (experimental group) or an equal volume of saline (control and blank groups). Experimental and control group mice were subjected to EP.

Establishment of the GD mouse model. Mice were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Shanghai Haling Biotechnology Co., Ltd., Shanghai, China). At weeks 1, 4, 7 and 10, recombinant plasmid pcDNA3.1/TSHR268 (50 μl, 1 g/l) was injected into the bilateral gastrocnemius of experimental group mice. An equal volume of saline was injected into the control and blank group mice at the same time. The experimental and control group mice were subjected to EP at the same time and location to enhance immunization. For EP, a pair of electrode needles was injected into the muscle across the site at which the recombinant plasmid was injected. Electric pulses were delivered using an electroporator. A total of three 50 V pulses and three additional pulses of the opposite polarity were administered at each injection site at a rate of one pulse per 200 msec, a frequency of 5 pulses/sec and a duration of 50 msec (10).

T₄ and TSH measurements. Blood from the angular vein was collected to measure the levels of total serum T₄ and serum TSH using radioimmunoassay and immunoradiometric kits, respectively (Beijing North Institute of Biotechnology, Beijing, China) 7 to 10 days after each immunization and every three weeks until week 21.

ELISA for measuring TRAb N-terminal (N) and C-terminal (C). Blood from the angular vein was collected to measure the levels of TRAb N and C by ELISA at the same time points as above. Thyroid-stimulating antibody (TSAb) was used in the N method and thyroid stimulation blocking antibody (TSBAb) was used in the C method, as respective antigens. A total of two ELISA methods for the detection of TSAb and TSBAb have been established and evaluated by our laboratory (11). The absorbance value A₄₅₀ served as the quantitative parameter to reflect the levels of TRAb. TRAb C levels were measured in arbitrary units per milliliter (AU/ml) (12).

^{99m}TcO₄⁻ radioactive isotope imaging. Whole body ^{99m}TcO₄⁻ imaging was performed at weeks 0, 12 and 21. A total of three mice from each group were randomly selected and anesthetized with pentobarbital. ^{99m}TcO₄⁻ was intraperitoneally injected into each mouse (37 MBq/0.1 ml). Scintigraphy was performed 10 min later using a SPECT machine. A low-energy universal collimator was used for imaging. The imaging conditions were as follows: Energy peak, 140 keV; matrix, 128x256; magnification, 3.0; and counts, 3x10⁵.

Weight, thyroid morphology and pathology analysis. All mice were weighed 7 to 10 days following each immunization and every three weeks until week 21. They were subsequently sacrificed by cervical vertebra dislocation at the end of

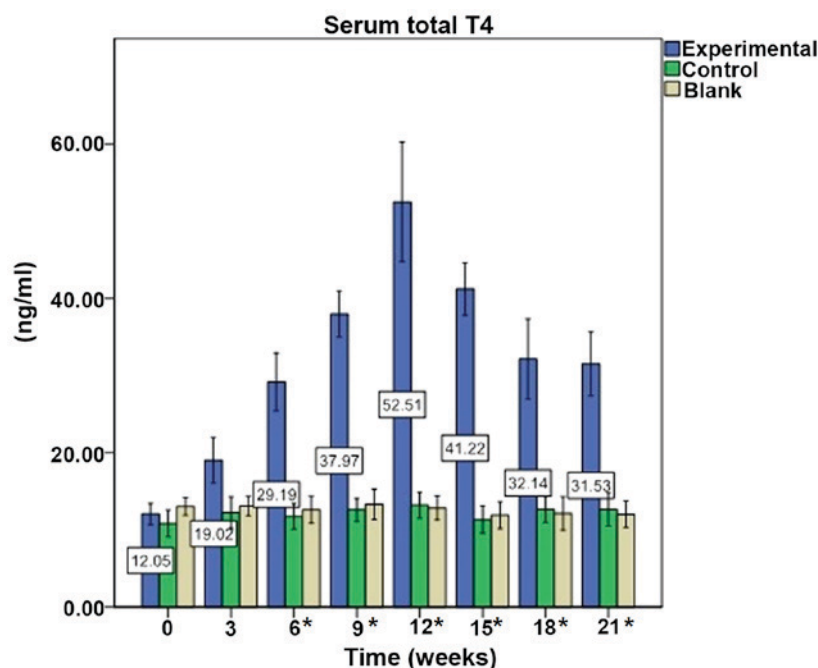


Figure 1. Immunization elevates total serum T₄ levels. Prior to immunization, mean levels of T₄ in the experimental, blank and control groups were similar. Following immunization, mean levels of T₄ in experimental mice increased gradually. Upon cessation of immunization, T₄ levels in the experimental group decreased slightly; however, they remained significantly elevated compared with pre-immunization levels. There were no significant alterations in T₄ levels in the control and blank groups during the experimental process. Data are presented as the mean ± standard deviation. *P<0.05. T₄, thyroxine.

week 21. The thyroid glands were removed and fixed with 10% formalin. After one week, the tissue was embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin for analysis (13).

Statistical analysis. Data were analyzed using an independent samples t-test or one-way analysis of variance followed by a least significant difference post hoc test. SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA) was used to analyze the data. Data are expressed as the mean ± standard deviation or number (%). P<0.05 was considered to indicate a statistically significant difference.

Results

Induction of GD in mice. During the course of the experiment, two mice in the experimental group and one in the blank group died. Prior to the experiment, T₄, and TRAb N and C levels of all mice in the experimental group were within the same range as those in the control and blank groups. At the end of the immunization protocol (week 12), 38 of the 48 mice in the experimental group had increased levels of T₄, and TRAb N and C. All 38 mice demonstrated reduced levels of TSH. The rate of disease induction was therefore 79.17%.

Immunization elevates serum T₄ levels. Prior to immunization (week 0), baseline mean levels of T₄ in the experimental mice were 12.05±4.23 ng/ml. Following the first immunization, the mean levels of T₄ increased slowly and peaked following the fourth immunization at week 12, where T₄ was 52.51±23.58 ng/ml (P<0.0001). T₄ levels decreased from weeks 15 to 21; however, they remained significantly increased

compared with pre-immunization levels (all P=0.000). T₄ levels in the control group were 13.21±3.73 ng/ml at week 12 vs. 10.84±3.79 ng/ml at week 0 (P=0.593), and in the blank group were 12.84±3.36 ng/ml at week 12 vs. 13.03±2.47 ng/ml at week 0 (P=0.892). There were no significant alterations in T₄ levels in the control and blank groups during the experiment (Fig. 1).

Immunization reduces serum TSH levels. Prior to immunization (week 0), baseline mean levels of serum TSH in the experimental mice were 5.53±2.78 μIU/ml. Following the first immunization, the mean levels of TSH declined (all P<0.0001). During the course of the experiment, TSH levels fluctuated; however, they remained reduced compared with pre-immunization levels (all P<0.0001). TSH levels in the control group were 5.35±2.17 μIU/ml at week 12 vs. 5.16±2.70 μIU/ml at week 0 (P=0.969), and in the blank group were 5.25±2.72 μIU/ml at week 12 vs. 5.10±1.99 μIU/ml at week 0 (P=0.960). There were no significant alterations in serum TSH levels in the control and blank groups (Fig. 2).

Immunization elevates TRAb N and C levels. Prior to immunization (week 0), baseline mean levels of serum TRAb N and C in experimental mice were 0.006±0.002 mIU/ml and 11.111±2.808 AU/ml, respectively. Following the first immunization, the mean levels of TRAb N and C increased steadily. Following the third immunization at week nine, TRAb N and C levels peaked at 0.306±0.146 mIU/ml and 51.566±37.357 AU/ml, respectively (all P<0.0001). From the fourth immunization (week 12) to the end of the experiment (week 21), the mean levels of TRAb N and C decreased; however, they remained significantly increased compared with pre-immunization levels (TRAb C at week 21, P=0.001,

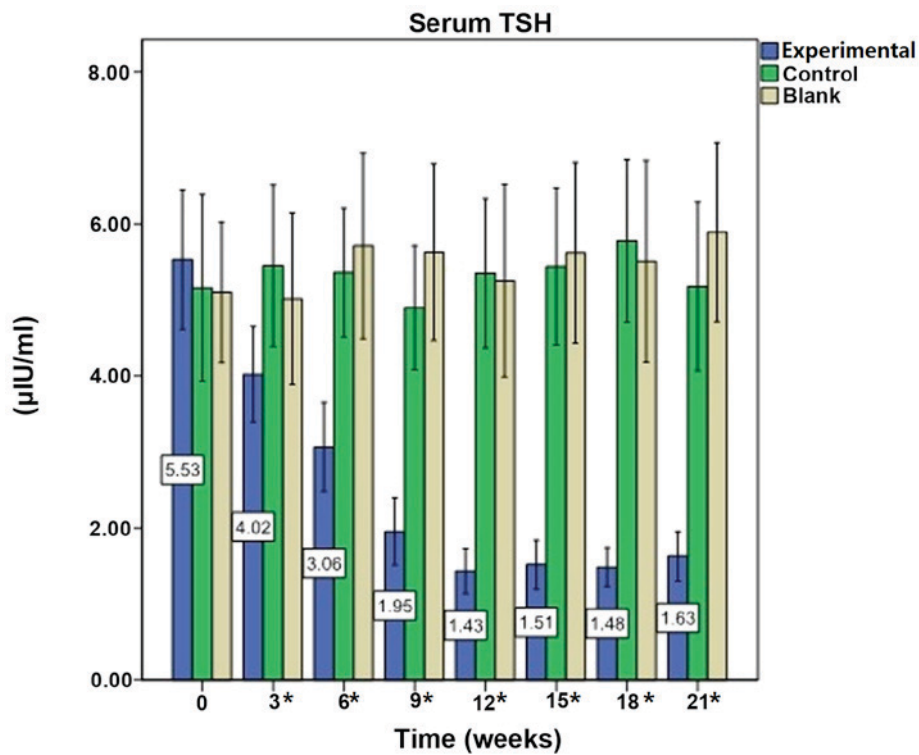


Figure 2. Immunization reduces serum TSH levels. Prior to immunization, mean levels of serum TSH in the experimental, blank and control groups were similar. Following the first immunization, mean levels of TSH in experimental mice reduced until the fourth immunization at week 12. TSH levels fluctuated following cessation of immunization; however, they remained reduced compared with pre-immunization levels. There were no significant alterations in serum TSH levels in the control and blank groups. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. TSH, thyroid-stimulating hormone.

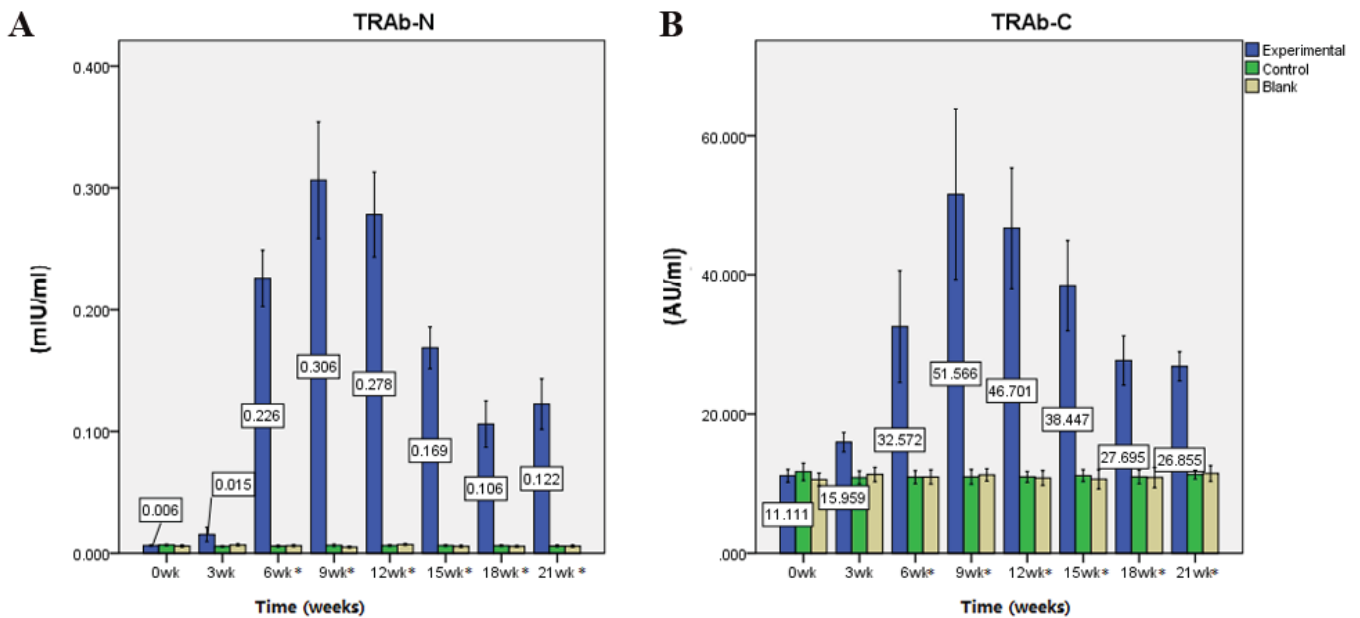


Figure 3. Immunization elevates TRAb N and C levels. (A) Mean levels of TRAb N, the thyroid-stimulating hormone receptor-stimulating antibody, markedly increased in the experimental group compared with the control and blank groups following immunization at week six. (B) Mean levels of TRAb C increased synchronously with TRAb N; however, at a more gradual rate. Following the third immunization at week nine, TRAb N and TRAb C levels peaked. Upon cessation of immunization, mean levels of TRAb N and C decreased; however, they remained significantly increased compared with pre-immunization levels. There were no significant alterations in the control and blank groups during the experimental process. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. TRAb N, thyroid-stimulating hormone receptor N-terminal; TRAb C, thyroid-stimulating hormone receptor C-terminal.

the rest P<0.0001). TRAb N levels in the control group were 0.006±0.002mIU/mlatweek9 vs.0.007±0.002mIU/mlatweek 0 (P=0.520), and in the blank group were 0.005±0.002 mIU/ml

at week 9 vs. 0.006±0.002 mIU/ml at week 0 (P=0.885). TRAb C levels in the control group were 10.97±2.35 AU/ml at week 9 vs. 11.69±2.78 AU/ml at week 0 (P=0.933), and

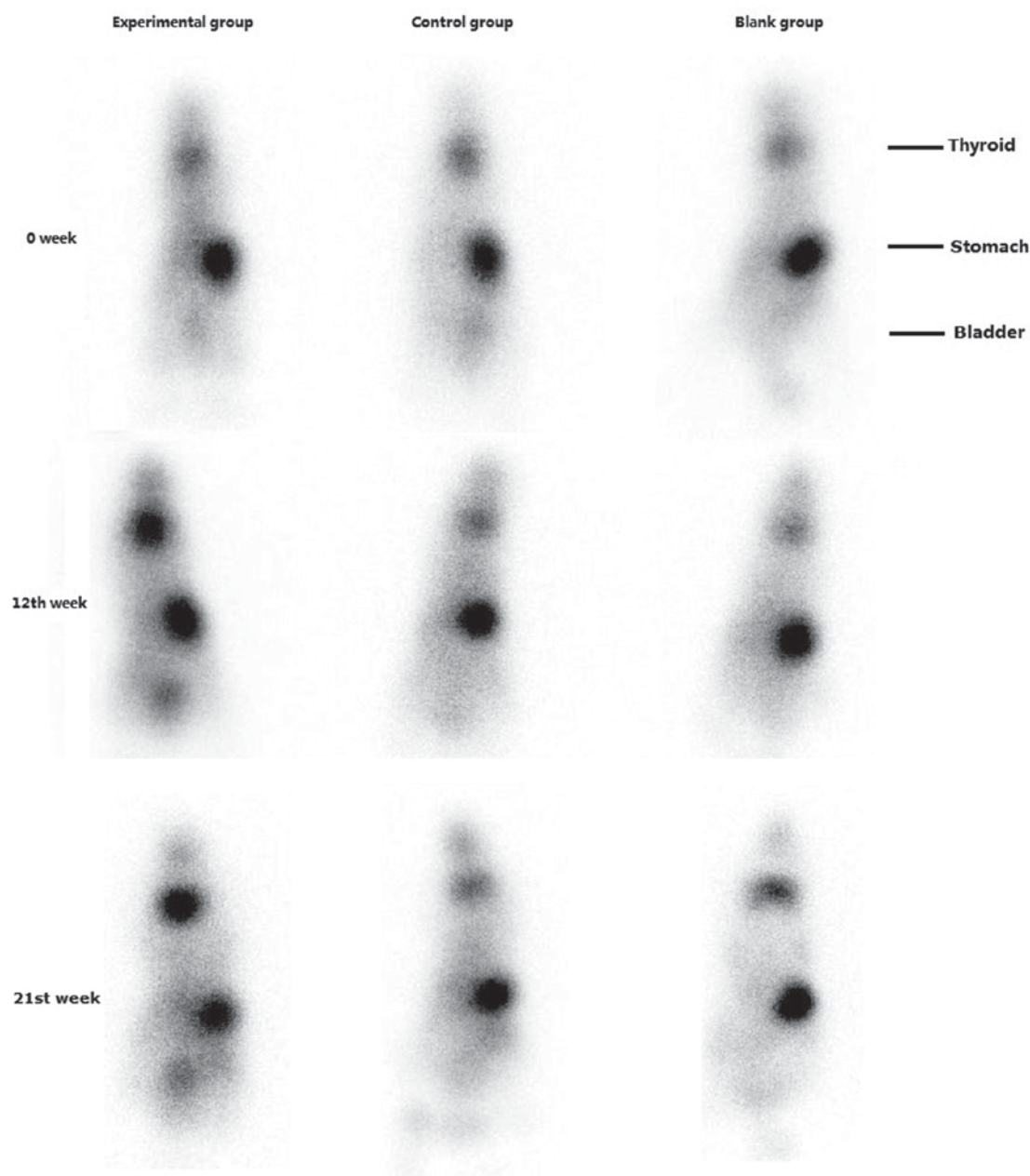


Figure 4. $^{99m}\text{TcO}_4^-$ radioactive isotope imaging. Prior to immunization at week 0, the uptake of $^{99m}\text{TcO}_4^-$ by the thyroid was similar between the experimental, blank and control group mice. Following four immunizations (at week 12), increased uptake of $^{99m}\text{TcO}_4^-$ by the thyroid was observed in the experimental group. At week 21, this uptake remained increased compared with pre-immunization levels. There were no significant alterations in $^{99m}\text{TcO}_4^-$ uptake in the control and blank groups. $^{99m}\text{TcO}_4^-$, pertechnetate.

in the blank group were 11.25 ± 1.91 AU/ml at week 9 vs. 10.59 ± 1.99 AU/ml at week 0 ($P=0.939$). There were no significant alterations in TRAb N and C levels in the control and blank groups (Fig. 3).

$^{99m}\text{TcO}_4^-$ radioactive isotope imaging. There were no significant differences in thyroid $^{99m}\text{TcO}_4^-$ uptake between the three groups prior to immunization. Following the fourth immunization (week 12), the uptake of $^{99m}\text{TcO}_4^-$ by the thyroid was increased in the experimental group. At week 21, this remained increased compared with pre-immunization levels. There were no significant alterations in $^{99m}\text{TcO}_4^-$ thyroid uptake in the control and blank groups (Fig. 4).

Weight, thyroid morphology and pathology analysis. At week 0, the mean weights of mice in the three groups were similar. The weight of mice increased steadily during the experiment in all groups. At week 21, the mean weight of the experimental mice was significantly reduced compared with the blank and control groups (Table I).

The thyroid glands from experimental group mice exhibited diffuse enlargement with hypertrophy, unlike the blank and control mice (Fig. 5). Examination of the thyroid tissue via microscopy revealed lymphocyte infiltration, fewer colloid nodules and an increase in the height of epithelial cells in experimental mice compared with blank and control group mice (Fig. 6).

Table I. Weight analysis.

Group	Number of mice	Weight of mice (g)									
		Week 0	Week 3	Week 6	Week 9	Week 12	Week 15	Week 18	Week 21		
Experimental	38	20.20±1.48	20.74±1.38	21.45±1.70 ^b	20.83±1.87 ^{a,b}	20.78±1.29 ^{a,b}	21.13±2.61 ^{a,b}	21.92±1.57 ^{a,b}	21.84±2.28 ^{a,b}		
Control	20	20.73±1.31	20.23±1.51	21.96±1.13	22.34±1.92	24.45±1.27	24.76±1.86	25.90±1.65	25.46±1.45		
Blank	19	20.89±1.36	20.31±1.88	22.04±1.62	22.88±1.95	24.23±1.59	24.27±1.40	25.40±1.64	25.36±1.90		

Prior to immunization, the mean weight of the three groups of mice was similar. During the process of the experiment, the weight was observed to increase in all groups. However, the mean weight gain of the experimental group was not significant. At the end of the experiment, the rate of weight increase in control and blank groups was significantly greater compared with the experimental group (25% vs. 5%). Data are presented as the mean ± standard deviation, ^aP<0.05 vs. control group, ^bP<0.05 vs. blank group.

Discussion

The immunological processes involved in autoimmune thyroid disease, including GD, are hypothesized to be mediated primarily by antibodies, similarly to other autoimmune diseases (14). Hyperactivity of the thyroid gland is the result of thyroid-stimulating antibodies, which are now considered to recognize and activate the TSHR. One of the unique characteristics of GD is the presence of TRAb, which is detectable in the serum of the majority of patients with the disease, and leads to hyperplasia and hyperfunctioning of the thyroid gland due to activation of the thyrotropin receptor (4,5,15). Our previous study demonstrated that, as one of the most important risk factors for GD, TRAb influenced prognosis and treatment efficacy, particularly in female patients (16). A total of three varieties of TRAb are present in most GD patients: TSAb, TSBAb and a neutral antibody. TSAb and TSBAb bind to the N- and C-terminal regions of the TSHR extracellular domain, respectively. It is hypothesized that TSAb serves a central role in the pathogenesis of GD, by stimulating TSH-mediated activation of thyroid function. However, TSBAb may act as a weak TSH agonist to induce hypothyroidism by preventing TSH from binding to the receptor. The neutral TSHR antibody does not block TSH binding or action (17). Previous studies have indicated that TSBAb may be the precursor form of TSAb (18).

The establishment of an animal model of GD contributes to the investigation of its etiology, pathogenesis and potential therapeutic targets. Following decades of developments, animal models of GD have greatly improved. However, limitations of GD mouse models include the complex model preparation process, low success rate and poor reproducibility. An ideal GD mouse model should be spontaneous, reproducible and have a high disease incidence in a variety of mouse strains (19). Thus far, the common features of all successful GD models include TSHR expression, immune system stimulation via repeated injections.

In the present study, following four immunizations, 38 of 48 BALB/c mice (79.17%) developed hyperthyroidism, a percentage that is consistent with or greater than previous studies (20-23). All hyperthyroid mice demonstrated increased serum T₄, TRAb N and C levels, and reduced TSH and weight levels. It was observed that TRAb N levels markedly increased following the second immunization at week 6, and peaked at week 9. TRAb C levels demonstrated a similar trend; however, the increase was more gradual. Under the stimulation of TRAb, serum T₄ levels increased gradually and peaked following the fourth immunization at week 12. Over the same period, serum TSH levels declined gradually. Following the fourth immunization, serum TSH levels stabilized. Following cessation of immunization, serum T₄ and TRAb levels decreased slightly; however, at the end of the experiment, they remained significantly increased compared with pre-immunization levels. The uptake of ^{99m}TcO₄⁻ by the thyroid increased significantly in immunized mice. Compared with the control and blank groups, mice in the experimental group were emaciated and exhibited enlarged thyroid glands. Examination of the thyroid tissue via microscopy revealed lymphocyte infiltration, fewer colloid nodules and an increase in the height of epithelial cells in the experimental mice compared with the blank and control groups. All of the above are characteristic of GD, thus

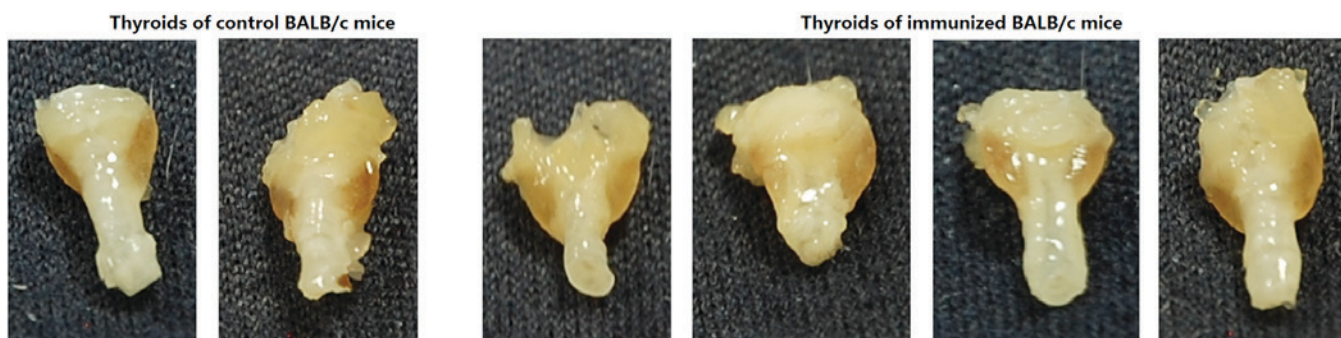


Figure 5. Thyroid morphology analysis. Representative images of the thyroids of control and immunized BALB/c mice. Following immunization, the thyroid glands from hyperthyroid mice were enlarged compared with the thyroid glands from control mice, a feature that may have been induced by the thyrotrophin receptor antibody.

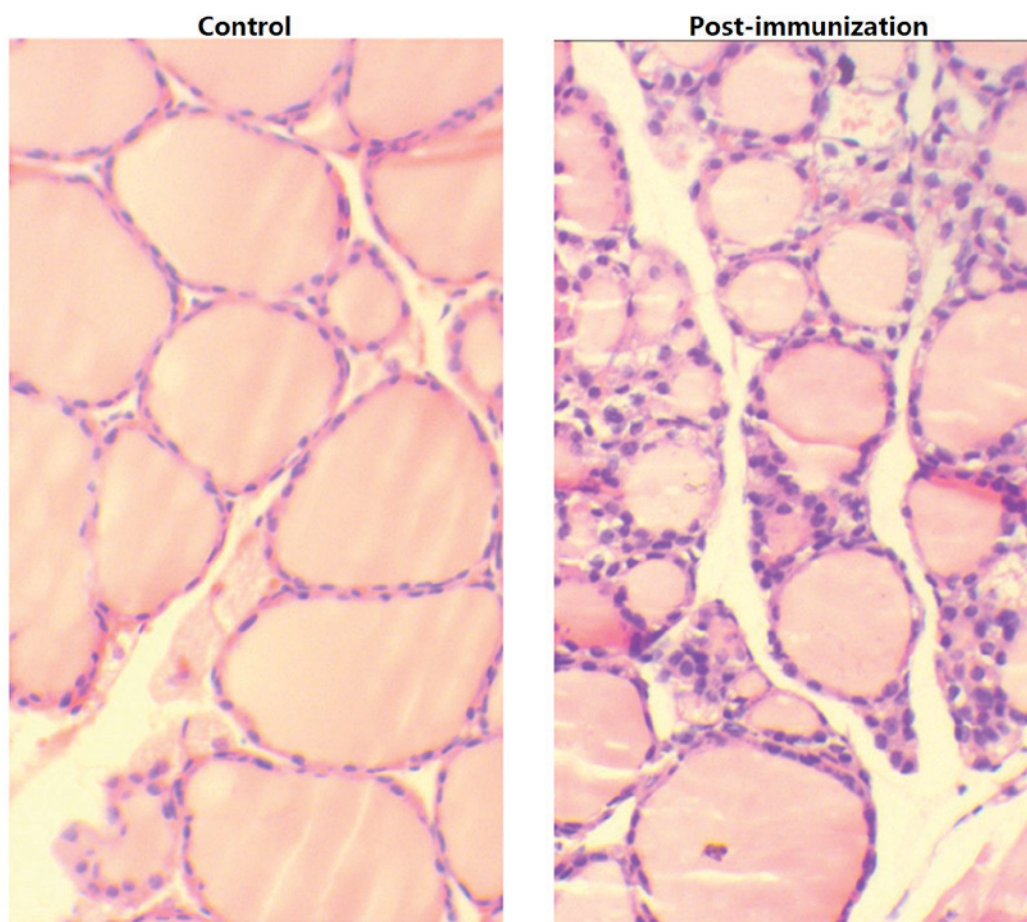


Figure 6. Thyroid pathology analysis. Frozen sections of the thyroid glands from control and immunized mice were stained with hematoxylin and eosin. Examination of the thyroid tissue via microscopy revealed lymphocyte infiltration, fewer colloid nodules and an increase in the height of epithelial cells in experimental compared with control mice. Magnification, x200.

indicating the establishment of a successful model of the disease. Furthermore, the increased levels of TRAb C support the hypothesis that TSBA b may be the precursor form of TSAb.

It is widely accepted that the study of animal models of disease provides an improved understanding of the underlying pathogenesis, and may be used for evaluating novel therapeutic strategies. An appropriate animal model of GD would include the following features: i) Elevated T_4 and/or reduced TSH levels, ii) TSHR-associated biologically active antibodies,

iii) alterations in thyroid architecture, iv) lymphocytic thyroiditis, v) clinical signs of hyperthyroidism including weight loss, vi) orbital alterations similar to those in thyroid eye disease, and vii) lymphocytic infiltration in the pretibial skin (24). With the exception of pathological alterations of eyes and pretibial skin, the additional five clinical characteristics were observed in the model established in the present study.

In conclusion, the present study demonstrated an improved animal model of GD in which repeated intramuscular injection

of recombinant plasmid pcDNA3.1/TSHR268 and EP *in vivo* efficiently induced TRAb with thyroid stimulating activity and hyperthyroidism. This novel method has the advantages of a short modeling cycle, high induction rate, and good stability and reproducibility. This model may not precisely mimic human disease, and therefore requires further examination; however, based on these findings, it may be possible to further investigate the etiology and pathogenesis of GD, and develop novel ideas for the clinical treatment of this disease.

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