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Immunotherapy with a biologically active ICAM-1 mAb and an siRNA targeting TSHR in a BALB/c mouse model of Graves' disease

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Abstract

Background: The objective was to study targeted therapies using a biologically active monoclonal antibody against intracellular adhesion molecule-1 (ICAM-1 mAb) and an siRNA targeting thyroid-stimulating hormone (TSH) receptor (TSHR) in a BALB/c mouse model of Graves' disease (GD).

Material and methods: An improved method for establishing a stable model of GD in BALB/c mice was developed by immunization with pcDNA 3.1/TSHR 289 and electroporation (EP). The mice in which GD was successfully established were divided into a nontreated control group, which was treated with continuous immunization, and treated groups, which were treated with the siRNA and ICAM-1 mAb. Normal mice were included as a blank group. These groups were used to compare the effects of treatment with the ICAM-1 mAb and siRNA.

Results: The two novel treatments markedly improved weight loss, serum thyroxine

(T_4) levels, thyroid-stimulating hormone antibody (TSAb) levels, thyroid-stimulating blocking antibody (TSBAb) levels and thyroid uptake of $^{99m}TcO_4$ in GD model mice. Compared with the siRNA treatment, treatment with the ICAM-1 mAb produced more obvious benefits. The differences in the posttreatment indexes between the two treatment groups were statistically significant ($p < 0.05$).

Conclusions: These preliminary data suggest that both the biologically active ICAM-1 mAb and the siRNA targeting TSHR were effective. The ICAM-1 mAb exerted a better therapeutic effect than the siRNA targeting TSHR. Both treatments showed potential efficacy as novel treatments for GD and may therefore represent therapeutic options in addition to the existing drugs or interventions.

Key words: Graves' disease; Graves' ophthalmopathy; TSHR; siRNA; monoclonal antibody; ICAM-1

Introduction

Graves' disease (GD) is an autoimmune thyroid disorder that is caused by antibodies directed against the TSH receptor (TSHR), leading to hyperthyroidism, and a genetically predisposed autoimmune disease. Typical characteristics of GD include metabolic syndrome, goiter and Graves' orbitopathy (GO). The condition is generally mediated by autoantibodies but also involves subsets of immune cells [1].

Currently, three main treatment methods for GD are used in clinical practice: antithyroid drugs (ATDs), radioactive iodine (RAI) therapy and surgical treatment. However, ATD treatment is characterized by relatively high relapse rates and concerning side effect profiles, and the other two treatments may cause hypothyroidism [2]. The current treatment options for GD are also suboptimal because they target the signs and symptoms of the disease rather than the pathogenic mechanisms. If left untreated, GD leads to significant morbidity and mortality [3].

TSHR is currently the main focus of research on the etiology and pathogenesis of GD, and TSHR is an important independent risk factor in the pathogenesis of GD [4]. Many scholars [5–7] postulate that the thyrotropin receptor antibody (TRAb), a self-

reactive antibody against TSHR, binds to TSHR on the thyroid cell membrane to cause thyroid cell stimulation, excessive growth and thyroid hormone synthesis. Increased hormone secretion leads to thyrotoxicosis [8]. The duration of ATD treatment is affected by the titer of TRAb [9]. Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily that is expressed on the plasma membrane of antigen-presenting cells. ICAM-1 plays an important role in the development of autoimmune thyroid disease and GO [10, 11]. The level of soluble ICAM-1 in the serum is related to GO activity and severity and can be used as an indicator for the GO diagnosis, staging, disease monitoring and treatment efficacy evaluations [12]. In our paper, the treatment strategies used to treat GD model mice included a biologically active ICAM-1 mAb and an siRNA targeting TSHR, which directly act on ICAM-1 and TSHR, respectively.

Material and methods

Establishment of the GD mouse model

A total of female BALB/c mice were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). They were all 6 to 8 weeks old and weighed 18 to 20 g. All animals were housed with free access to laboratory food and water on a 12/12 h light/dark cycle. They were housed in groups of 5 animals per cage at a constant temperature (18–20°C) and humidity (65–69%). pcDNA 3.1/TSHR 289 was prepared and preserved in our laboratory. All 70 female BALB/c mice were injected with pcDNA 3.1/TSHR 289 into the bilateral gastrocnemius and electroporated (EP) every 3 weeks 4 times. The protocol for establishing the GD mouse model was described in our previous article [13]. This work was approved by the Ethics Committee of Tianjin Medical University General Hospital (NO. IRB2020-DW-12).

Cellular siRNA experiment

Nthy-ori-3-1 normal human thyroid cells (No. c1002) were purchased from Huiying Biological Technology Co., Ltd. (Shanghai, China). The cells were cultured in a cell incubator at 37°C with 5% CO_2. According to the TSHR gene sequence published by GenBank (Rat GI 6981679, Mouse GI 575923), two siRNAs were designed and synthetized in accordance with the homologous gene sequence of TSHR in Nthy-ori-3-1 normal human thyroid cells and BALB/c mice using Ambion's online design software. The siRNA sequences were as follows: siRNA 1012: sense ACAAAACUCCAAGUUCCAGtt, antisense CUGGAACUUGGAGUUUUGUtt; and siRNA 1041: sense GCAACUCUCACUAUUACGUtt, antisense ACGUAAUAGUGAGAGUUGCtt. Designed siRNAs were purchased from Bao Biotechnology Co., Ltd. (Dalian, China). Nthy-ori-3-1 cells were cultured with RPMI 1640 medium in an incubator, and synthetic siRNA was transfected into Nthy-ori-3-1 cells. Cellular proteins were extracted after transfection. The difference in the expression levels of TSHR before and after transfection was detected using Western blotting.

Preparation of the ICAM-1 mAb

ICAM-1 mAbs were prepared and preserved in our laboratory. We used pure ICAM-1 protein to immunize BALB/c mice, removed the spleen, and fused the cells with myeloma cells (SP2/0). We selected the secretion wells, subcloned them and prepared mAbs in quantity using the induction method in vivo. Then, we acquired the ICAM-1 mAb from purified ascites. The protocol for preparing the ICAM-1 mAb was published in our previous article [14].

Animal grouping and treatment plan

At week 12, which was the end of the immunization period, 53 of the 70 mice presented increased levels of T_4, TSAb and TSBAb. The rate of immunization success was therefore 75.71%. Fifty mice in which GD was successfully established were

randomly assigned to five groups (groups A–E). In addition, age-matched mice that were unimmunized and untreated were used as a control for comparisons (group F). Each group included 10 mice.

The study protocol included four immunizations every 3 weeks (groups A-E), followed by a 'maintenance' phase that included additional regular immunizations every 3 weeks until the end of the experiment (groups A, C, and E). The siRNA (10 μg/piece) was administered to groups A and B via intraperitoneal (i.p.) injection 3 times 2 weeks after the fourth immunization (week 12), with 2 days between each injection. The ICAM-1 mAb (10 μg/injection) was administered to groups C and D using the same method as the siRNA was administered to groups A and B. The siRNA and ICAM1 mAb were dissolved in 0.9% NaCl without further additives. A detailed treatment schedule is shown in Figure 1. A detailed explanation of the animal groups is shown in Table 1.

Serum indicator measurements

Total serum T_4 levels were measured in blood collected from the angular vein using radioimmunoassay and immunoradiometric kits (Beijing North Institute of Biotechnology, Beijing, China) at 2 weeks after each immunization and at the end of the experiment. TSAb and TSBAb levels were measured in blood collected from the angular vein using ELISAs at the same time points described above. Two ELISA methods for the detection of TSAb and TSBAb have been established and evaluated by our laboratory [15].

$^{99m}TcO_4^-$ radioactive isotope imaging

The mouse images were obtained by using a small animal single-photon emission computer tomography (SPECT) machine (NanoSCAN; Mediso, Hungary). Pertechnetate ($^{99m}TcO_4^-$) was produced by a molybdenum technetium generator (China Institute of Atomic Energy, Beijing, China). Whole-body $^{99m}TcO_4$ imaging was performed at week 12 (2 weeks after the fourth immunization) and week 18 (the end

of the experiment). Each mouse was anesthetized with 3% isoflurane, followed by an intraperitoneal injection of 37 MBq $^{99m}TcO_4^-$ (1 mCi/0.1 mL) while the mouse was in a prone position. The scintillation scan was performed 10 min later using the NanoSCAN small animal SPECT/CT with a multipinhole collimator, peak energy of 140 keV, and total acquisition time of only 2 h.

Weight, morphological and pathological analyses

A total of mice were weighed 2 weeks after each immunization and at the end of the experiment. The mice were subsequently sacrificed by cervical vertebra dislocation at the end of the experiment. The thyroid gland, eyeball and heart were removed and fixed with 10% formalin. One week later, the tissues were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (HE) for analysis, and pathological changes were observed.

Statistical analysis

SPSS software version 25.0 (IBM SPSS, Armonk, NY, USA) was used to analyze the data. The data from each group are presented as the means±standard deviations. The t test was used to compare means between two groups, and the LSD method with one-way analysis of variance (one-way ANOVA) was used to compare means between multiple groups. $p < 0.05$ was considered to indicate statistical significance.

Results

TSHR expression after siRNA transfection

Twenty-four hours after the siRNA transfection, the expression level of TSHR in the Nthy-ori-3-1 cells was significantly downregulated (shown in Fig. 2).

Determination of serum T_4 levels

The T_4 levels did not differ between experimental mice before immunization (week 0), and the average levels of T_4 increased slowly. After initiating therapy, the two treatment groups showed progressively decreasing T_4 levels. Among the sustained

immunization groups, the T_4 levels in the siRNA group (group A) decreased from 65.71 ± 6.89 ng/mL to 27.58 ± 1.94 ng/mL, and those in the ICAM-1 mAb group (group C) decreased from 70.84 ± 8.46 ng/mL to 27.24 ± 3.50 ng/mL. Among the groups without sustained immunization, the T_4 levels in the siRNA group (group B) decreased from 68.23 ± 6.38 ng/mL to 22.48 ± 2.43 ng/mL, and those in the ICAM-1 monoclonal antibody group (group D) decreased from 67.45 ± 7.29 ng/mL to 16.32 ± 1.53 ng/mL (all $p < 0.05$). However, the values mentioned above were still significantly higher than those in the unimmunized blank group (group F) (13.97 ± 1.41 ng/mL) and lower than those in the immunized group (group E) (69.03 ± 2.72 ng/mL) at the same time points before and after treatment ($p < 0.05$ shown in Fig. 3).

Determination of serum TSAb and TSBAb levels

The mean levels of TSAb and TSBAb gradually increased following the first immunization. The decreases in the serum TSAb and TSBAb levels in the two treatment groups were compared. Among the sustained immunization groups, the TSAb and TSBAb levels in the siRNA group (group A) decreased from 457.33 ± 45.85 μIU/mL to 331.44 ± 43.38 μIU/mL and from 15.83 ± 5.92 μIU/mL to 13.94 ± 1.11 μIU/mL, respectively. The levels in the ICAM-1 mAb group (group C) decreased from 443.91 ± 42.32 μIU/mL to 275.16 ± 45.80 μIU/mL and from 17.05 ± 6.16 μIU/mL to 14.59 ± 1.02 μIU/mL, respectively (all $p < 0.05$). Among the groups without sustained immunization, the TSAb and TSBAb levels in the siRNA group (group B) decreased from 462.07 ± 46.12 μIU/mL to 283.91 ± 25.53 μIU/mL and from 15.22 ± 7.29 μIU/mL to 12.96 ± 1.27 μIU/mL, respectively. Levels in the ICAM-1 monoclonal antibody group (group D) decreased from 483.48 ± 44.29 μIU/mL to 119.12 ± 40.53 μIU/mL and from 16.81 ± 6.05 μIU/mL to 9.56 ± 1.99 μIU/mL, respectively (all $p < 0.05$). However, the values mentioned above were still significantly higher than those in the unimmunized blank group (group F) (6.37 ± 2.86 μIU/mL and 5.94 ± 3.22 μIU/mL, respectively) and lower than those in the immunized group (group E) (504.84 ± 42.77 μIU/mL and 17.14 ± 2.76 μIU/mL,

respectively) at the same time points before and after treatment ($p < 0.05$). After each treatment, a statistically significant decrease in the TSAb and TSBAb levels was observed in groups A-D (all $p < 0.05$No significant differences in the serum TSAb and TSBAb levels were observed in groups E and F during the course of treatment (shown in Fig. 4).

$^{99m}TcO_4^-$ radioactive isotope imaging

Thyroid $^{99m}TcO_4^-$ imaging was performed on each mouse (NanoSCAN small animal SPECT/CT, shown in Fig. 5). The uptake of $^{99m}TcO_4^-$ by the thyroid was increased in the experimental group following the fourth immunization. After treatment, compared with the blank group (group F), groups A-E exhibited increased $^{99m}TcO_4^-$ uptake in the thyroid. $^{99m}TcO_4^-$ uptake in the thyroid of group E was higher than that of the other groups. No significant differences in $^{99m}TcO_4^-$ uptake were observed between the two treatment groups.

Weight, morphological and pathological analyses

The mean weights of mice in the three groups were similar prior to the experiment. The weights of mice increased steadily during immunization. After treatment, the weights of the mice in each treated group (groups A-D) were slightly higher than those in group E (all $p < 0.05$) but still lower than those in the blank group (group F) (all $p < 0.05$). A significant difference in weight gain was not observed between any two treated groups (shown in Fig. 6).

As shown in Fig. 7, an examination of the thyroid tissues in groups A-E via microscopy revealed decreased thyroid follicular colloid and lymphocyte infiltration and partial microfollicular hyperplasia. Group F lacked these pathological changes. Optic nerve edema was observed in the partial postbulbar tissues of the mice (groups A–E) and was most obvious in group E. Edema of the extraocular muscles of mice was observed in group B. Group F lacked these pathological changes, but iris papillary hyperplasia was occasionally identified. HE staining of the heart tissues of

the mice in groups A–E showed endocardial fibroblast hyperplasia, dilation and congestion of blood vessels between the myocardium, but no significant differences in these pathological features were observed between the two treatment groups.

Discussion

Mouse models of GD allow us to better understand the disease and to investigate novel therapeutic approaches. Over the years, many scholars at home and abroad have tried to prepare GD animal models by using various methods, but the results have not been satisfactory [16]. Currently, the most common method for constructing a GD model is to immunize BALB/c mice with an adenovirus or plasmids expressing TSHR. Although this method achieves a high success rate and is easy to replicate, it also has some problems, such as a short expression time [17, 18]. Kim-saijo [19] attempted to establish a transgenic animal model, which transferred some TSAb gene fragments into the fertilized eggs of C57BL/6J mice. In the present study, an improved method was used to induce GD in BALB/c mice in which the recombinant plasmid pcDNA3.1/TSHR289 was injected followed by electroporation (EP). This model features an extension of the initial phase to include a maintenance phase. This novel method has the advantages of a high success rate, short model cycle, good stability, reproducibility and no interference from self-relief in model mice after the cessation of immunization.

GD is an autoimmune thyroid disease (AITD) characterized by hyperthyroidism, diffuse goiter and antibodies mainly against TSHR, thyroid peroxidase (TPO) and/or thyroglobulin (Tg) [20]. The current treatment options for GD are also suboptimal because they target the signs and symptoms of the disease rather than the pathogenic mechanisms. Scholars from all over the world are committed to finding a new treatment for the etiological cause of GD that will permanently treat this disease without destroying or removing the thyroid gland. Some scholars found that the presence of type 2 deiodinase peptide antibodies is associated with Graves' hyperthyroidism, influencing the levels of antibodies against TPO, Tg and TSHR, as

well as the therapeutic efficacy of antithyroid drugs [21]. According to Reyhane [22], TPO expression in the endometrium and placenta may explain the higher frequency of abortion and infertility in patients with thyroid autoimmunity. However, most of the research focuses on the development of inhibitors of TSHR, such as antibodies, and strategies to prevent GD by silencing genes. RNA interference (RNAi) refers to the introduction of a double-stranded RNA of a homologous sequence to an endogenous mRNA coding region into a cell to specifically degrade the mRNA and silence the expression of the gene. Although RNAi represents a fast and efficient technique for inhibiting gene expression, many scholars have found that siRNAs that are not randomly designed exert a gene-silencing effect. Song [23] designed a 6-segment siRNA corresponding to the gene encoding the mouse apoptotic receptor Fas, but only 3 of the siRNAs achieved the expected results. Yoshinari and colleagues [24] showed that the expected genomic silencing effect is substantially reduced when the starting position of the siRNA sequence is shifted by 1 to 2 bases. However, siRNA therapies are hindered by poor intracellular uptake, limited blood stability and nonspecific immune stimulation. In addition, the target cell types and target genes must be identified, and the temporal and spatial effects on gene expression must be understood to effectively utilize siRNA-mediated endogenous gene silencing in a therapeutic setting. In this experiment, the siRNA that was designed to inhibit the expression of the target gene TSHR and that exerted the best effect was selected for use as a treatment. The mRNA levels of the TSHR gene decreased significantly, and the levels of the TSHR protein decreased accordingly.

ICAM-1 is a cell surface single-chain glycoprotein that binds to lymphocyte function-associated antigen-1 ligand and mediates cell-to-cell, cell-to-matrix or cell-matrix-cell adhesion; ICAM-1 is involved in antigen recognition, complement fixation, and cell adhesion. ICAM-1 is also closely related to the pathogenesis of GD [25, 26]. Some scholars have confirmed that patients with GD exhibit a large amount of ICAM-1 expression on the surface of thyroid cells during continuous stimulation with autoantibodies. A large number of lymphocyte function-associated antigen-1

ligands are expressed on the membranes of infiltrating T lymphocytes and monocytes, and the ICAM-1/LFA-1 pathway mediates autoimmune damage to thyroid tissue [27]. Significant inhibition of the secretion and synthesis of soluble intercellular adhesion molecule-1 using glucocorticoids exerts a clear effect on GD by controlling the rate of disease development [28, 29]. Therefore, the level of sICAM-1 can be used as an important criterion to evaluate the severity of GD and to choose the time for intervention. The level of sICAM-1 can also be used to predict the risk of recurrence. Therefore, this study used the ICAM-1 monoclonal antibody previously constructed by our laboratory to treat GD model mice and explored its therapeutic effects [14]. The effects of the siRNA against TSHR and the ICAM-1 mAb were compared in the same study because these two treatment methods affect the immune state: TSHR-targeted therapy affects the cause of GD, ICAM-1-targeted therapy affects the immune state, and sICAM-1 therapy is a supplement to TRAb that affects the immune state and immune repair and can be used to evaluate the probability of disease recurrence. Therefore, both agents were studied together to prepare for subsequent studies of strategies using them in combination.

After the successful treatment of mice with the siRNA and ICAM-1 monoclonal antibodies, T_4 and TSAb levels were significantly decreased and the body weights increased. We showed that pcDNA 3.1/TSHR 289 immunization in mice can be extended using a novel protocol, in which regular injections every 4 weeks served to continuously increase antibody production. In summary, a long-term model of GD induced by a relatively simple protocol of sustained immunizations should facilitate the investigation of the mechanisms of long-term disease and may avoid the need for more complicated disease models. However, regardless of whether the recombinant plasmid was used for sustained immunization in the corresponding groups, serum T_4 levels were significantly improved compared to those in the untreated group, but they still did not reach normal levels. The uptake of thyroid $^{99m}TcO_4^-$ was lower in groups A-D than in group E, indicating that both treatment methods were effective. The serum T_4 and TSAb levels in the ICAM-1 monoclonal antibody-treated group were

lower than those in the siRNA-treated group, indicating that the therapeutic effect of the ICAM-1 monoclonal antibody was somewhat better than that of the siRNA. Many sites are present in the TSHR gene. In addition, siRNA therapies are limited by poor intracellular uptake, low blood stability and nonspecific immune stimulation. A relationship between the treatment dose and the duration of treatment has been identified. The most effective treatment dose and duration still require further exploration.

No significant difference in $^{99m}TcO_4^-$ uptake was observed between group A (continuous immunization + siRNA) and group C (continuous immunization + ICAM-1 mAb) or between group B (siRNA) and group D (ICAM-1 mAb). However, the $^{99m}TcO_4^-$ uptake in groups A-D was lower than that in group E, indicating that the two treatments were effective, but the low dose and short duration might affect the results observed after treatment. The pathological examination of mice in the late stage of the experiment revealed that the thyroid glands of the GD model mice showed corresponding hyperthyroidism characteristics, but a significant difference was not observed between the two treatments. Histological changes are often associated with serological changes, but histological changes after treatment usually occur later than serological changes. Optic nerve edema was observed in the partial postbulbar tissues of the mice and was most obvious in group E. Edema of the extraocular muscles of mice was detected in group B. We are not sure whether these symptoms are related to the exophthalmos observed in patients with hyperthyroidism, and further experimental data are needed to explain this phenomenon. Group F occasionally had visible iris papillary hyperplasia, but this change could have been related to the age of the mice. HE staining of the heart tissues of the mice in groups A-E showed endocardial fibroblast hyperplasia and dilation and congestion of blood vessels between the myocardium; fibroblast proliferation is usually related to thrombus. The abovementioned pathological changes may be related to heart diseases caused by hyperthyroidism, such as arrhythmia, which can cause thrombosis. However, this study has limitations. We cannot exclude the possibility that the

effective dose was not reached due to the short duration of treatment. In addition, we do not currently have a full explanation for the observed effects, which require further investigation.

In summary, treatment of a mouse GD model with an siRNA and ICAM-1 mAb led to marked improvements in several disease parameters. In our future research, we may extend the treatment time, use different treatment doses, increase the number of treatments appropriately, explore the best treatment methods and therapeutic doses, and examine the potential for future clinical treatment.

Conflicts of interest

The authors declare that they have no conflict of interest. Declarations of interest: none.

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Contribution to the field statement

Graves’ disease is an autoimmune thyroid disorder that is caused by antibodies directed against the TSH receptor, leading to hyperthyroidism, and a genetically predisposed autoimmune disease. The current treatment options for Graves’ disease are also suboptimal because they target the signs and symptoms of the disease

rather than the pathogenic mechanisms. Our research is trying to find the therapeutic target, fill the gaps in the etiological treatment of Graves' disease. Provide new ideas for the treatment and prevention of this disease.

Authors' contributions

X.W., W.L. — formal analysis, writing-original draft, writing-review and editing; Z.R. — formal analysis, writing-original draft; W.Z. — conceptualization, data curation, writing-review and editing; J.T. — conceptualization, writing-review and editing; N.L. — methodology, writing-review and editing; Y.Y. — investigation, writing-original draft

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Table 1. Animal grouping and treatment plan

	Immunized	Sirna	ICAM-1 MAB
Group A	+	+	-
Group B	-	+	-
Group C	+	-	+
Group D	-	-	+
Group E	+	-	-
Group F	-	-	-

Immunized — continuous recombinant plasmid pcDNA3.1/TSHR289 injection + EP; siRNA — intraperitoneal injection of siRNA (1 dose per 48h, 3 times total); ICAM-1 mAb — intraperitoneal injection of ICAM-1 monoclonal antibody (administered once every 48 h for 3 times)

Figure 1. A detailed treatment schedule

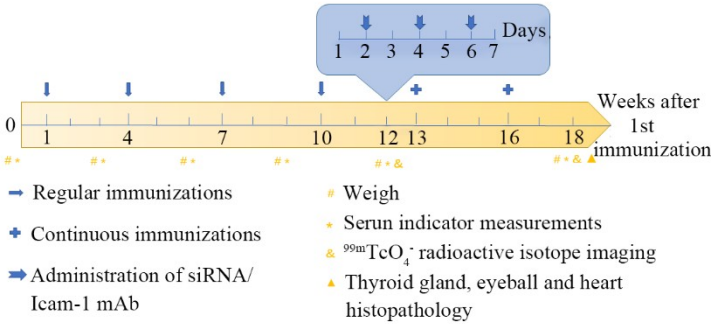

Figure 2. The expression level of TSHR in the Nthy-ori-3-1 cells. After siRNA transfection for 24 h, the expression level of TSHR in Nthy-ori-3-1 cells was significantly down-regulated

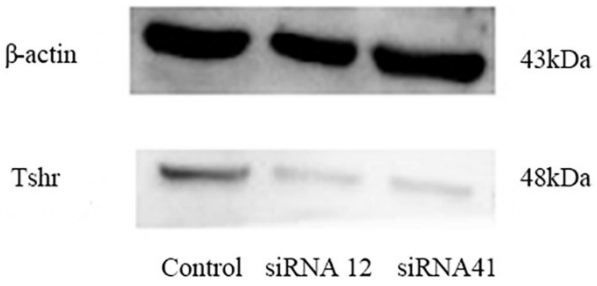

Figure 3. The levels of total serum T_4 before(lowercase) and after(uppercase) treatment. As can be seen, during continuous immunization, serum T_4 level decreased in siRNA group (group A) and ICAM-1 monoclonal antibody group (group C). During non-sustained immunization, serum T_4 level decreased in siRNA group (group B) and ICAM-1 monoclonal antibody group (group D). But at the same time point before and after treatment in the above group the above serum T_4 level still higher than the unimmunized blank group (group F) (#$p < 0.05$, all $p = 0.0001$) and lower than the immunized group (group E) (▲$p < 0.05$, all $p = 0.0001$). There was a statistically significant difference in T_4 levels of group A, group B, group C and group D before and after each their treatment (*$p < 0.05$, all $p < 0.0001$).

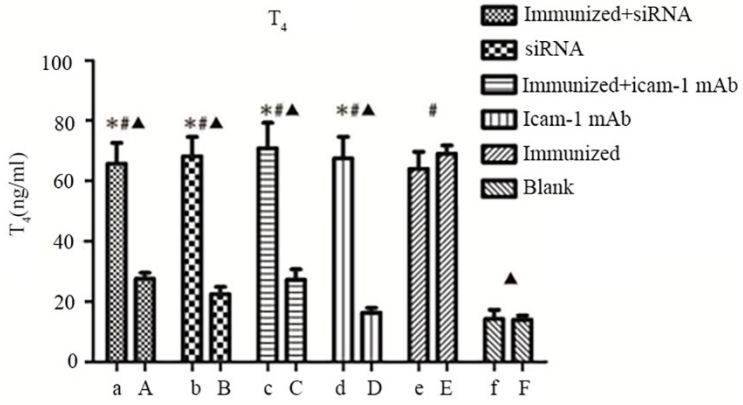

Figure 4. The levels of total serum TSAb and TSBAb before (lowercase) and after(uppercase) treatment. As can be seen, during continuous immunization, serum TSAb and TSBAb level decreased in siRNA group (group A) and ICAM-1 monoclonal antibody group (group C).During non-sustained immunization, serum TSAb and TSBAb level decreased in siRNA group (group B) and ICAM-1 monoclonal antibody group (group D).But the above data still higher than the unimmunized blank group (group F) (#$p < 0.05$, all $p = 0.0001$) and lower than the immunized group (group E) (▲$p < 0.05$, all $p = 0.0001$)at the same time point before and after treatment in the above group. There was a statistically significant difference in TSAb levels of group A, group B, group C and group D before and after each their treatment (*$p < 0.05$, all $p < 0.0001$)

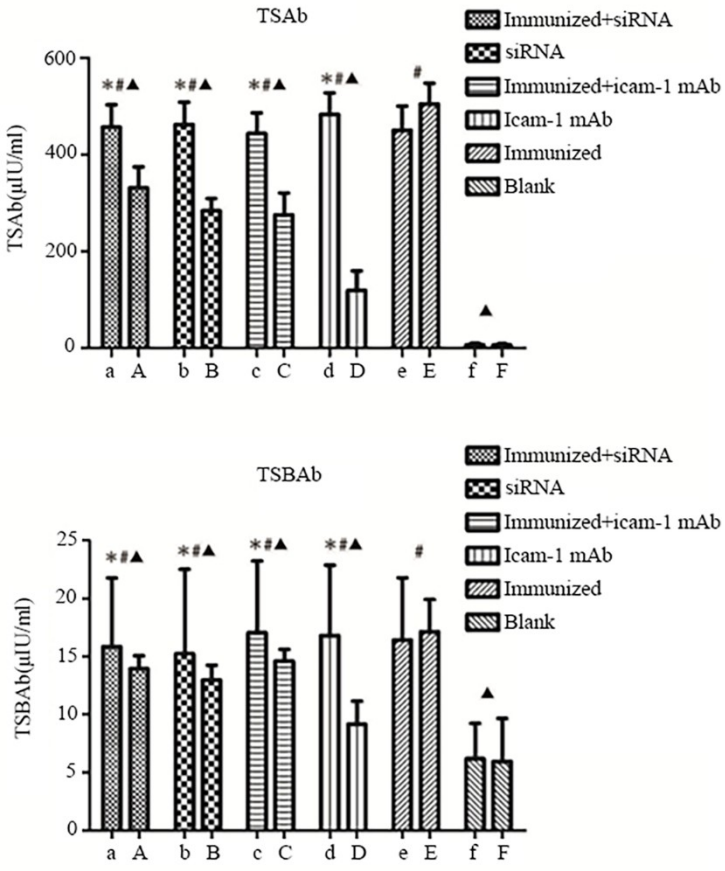

Figure 5. Thyroid $^{99m}TcO_4^-$ imaging after treatment. The tracer concentration region was observed as mouse thyroid. It can be seen that the uptake capacity of thyroid $^{99m}TcO_4^-$ in mice of group E is significantly enhanced compared with other groups. There were no significant differences in $^{99m}TcO_4^-$ uptake between the two treatment groups

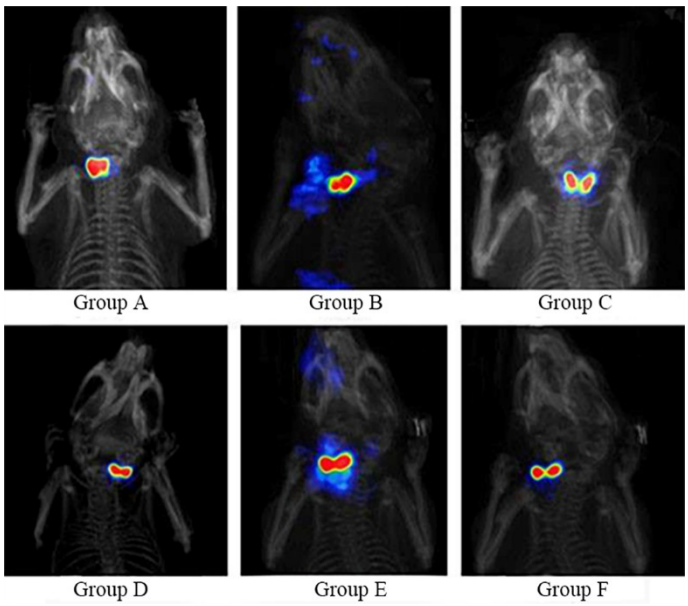

Figure 6. The weight of mice after treatment. It can be seen that the weight of mice in each group after treatment was slightly higher than that in the group E ($p < 0.05$, $p = 0.0464, 0.0222, 0.0344, 0.0283$), but still lower than the unimmunized F group ($p < 0.05$, $p = 0.0227, 0.0416, 0.0323, 0.0448$). There was no significant difference in the weight gain between the mice in each group

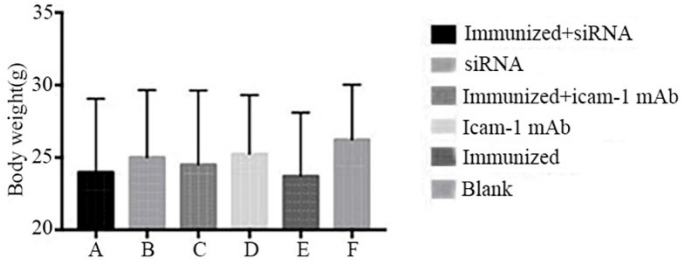

Figure 7. An examination of the thyroid tissues in groups A-E via microscopy revealed decreased thyroid follicular colloid and lymphocyte infiltration and partial microfollicular hyperplasia. Group F lacked these pathological changes. Optic nerve edema was observed in the partial postbulbar tissues of the mice (groups A–E) and was most obvious in group E. Edema of the extraocular muscles of mice was observed in group B. Group F lacked these pathological changes, but iris papillary hyperplasia was occasionally identified. HE staining of

the heart tissues of the mice in groups A–E showed endocardial fibroblast hyperplasia, dilation and congestion of blood vessels between the myocardium, but no significant differences in these pathological features were observed between the two treatment groups