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TSHR-based chimeric antigen receptor T cell specifically deplete auto-reactive B lymphocytes for treatment of autoimmune thyroid disease

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ABSTRACT

Graves' disease (GD) is a prominent antibody-mediated autoimmune disorder characterized by stimulating antibodies (TRAb) that target the thyroid-stimulating hormone receptor (TSHR). Targeting and eliminating TRAb-producing B lymphocytes hold substantial therapeutic potential for GD. In this study, we engineered a novel chimeric antigen receptor T cell (CAR-T) therapy termed TSHR-CAR-T. This CAR-T construct incorporates the extracellular domain of the TSH receptor fused with the CD8 transmembrane and intracellular signal domain (4-1BB). TSHR-CAR-T cells demonstrated the ability to recognize and effectively eliminate TRAb-producing B lymphocytes both in vitro and in vivo. Leveraging this autoantigen-based chimeric receptor, our findings suggest that TSHR-CAR-T cells offer a promising and innovative immunotherapeutic approach for the treatment of antibody-mediated autoimmune diseases, including GD.

1. Introduction

Chimeric antigen receptor (CAR) T cell (CAR-T) therapy is a promising immunotherapeutic approach involving the genetic engineering of T cells with a CAR fusion protein capable of recognizing specific antigens on target cells and exerting cytotoxic effects without major histocompatibility complex (MHC) restriction [1]. This groundbreaking therapy has shown great potential in the treatment of malignant tumors, particularly in B lineage hematological malignancies and multiple myeloma [2–5].

Beyond its successful application in cancer, recent research has explored the potential uses of CAR-T cells in treating autoimmune diseases, such as colitis, systemic lupus erythematosus, pemphigus vulgaris, and type 1 diabetes [6–11]. Currently, most treatments for autoimmune diseases rely on general immune suppression, which can increase the risk of infections for patients [12,13]. In contrast, CAR-T therapy offers a unique advantage by specifically eliminating pathogenic autoimmune cells while promoting protective immunity, making it an attractive therapeutic option for autoimmune diseases. Autoantigen-based CAR-T cells can directly target and kill auto-reactive B lymphocytes through targeted recognition. Notably, CAR-T cell therapy surpasses monoclonal antibodies, like rituximab, due to its ability to generate migration to lymphatic nodules, fostering the development of effective and memory cells with just a single application [14]. Consequently, CAR-T cells hold the potential to outperform monoclonal antibody therapy in terms of specific targeting, reduced adverse effects, and possible contribution to the permanent restoration of immune balance [15–18], especially in antibody-mediated autoimmune diseases.

One such autoimmune disease is Graves' disease (GD), a prototypical antibody-mediated disorder caused by stimulating antibodies that recognize the thyroid-stimulating hormone receptor (TSHR), known as TRAb. TRAb constitutes characteristic and pathogenic autoantibodies in Graves' disease [25]. In patients with Graves' disease (GD), a mix of monoclonal antibodies targeting various TSHR epitopes and polyclonal antibodies results in diverse clinical symptoms. GD often leads to hyperthyroidism, marked by increased T3 and T4 levels and notable goiter. The activating TRAb-M22 antibodies induce hyperthyroidism, while inhibitory ones like TRAb-(K1-70), TRAb-5c9, and TRAb-(K1-18) cause hypothyroidism. Despite hyperthyroidism being more common in GD, treatments directly addressing its root cause are limited. Hence, the specific elimination of TRAb-producing B lymphocytes through CAR-T therapy emerges as a potential and innovative therapeutic strategy for

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GD. Nevertheless, these strategies have faced challenges as the functionality of these cells has been limited by off-target attacks on healthy tissues, and specific cytotoxicity towards antibody-producing plasma cells remains unclear [19]. To address these obstacles, researchers have explored various cell therapy approaches in antibody-mediated autoimmune diseases using CAR-T cells, CAR-Tregs, and CAAR-T cells [11,14,20–23].

In this study, our goal was to target TRAb and develop TSHR-CAR-T cellsbased on the extracellular domain of TSHR. T cells carrying this chimeric receptor should be capable of eliminating TRAb-producing cells as long as they continue to secrete TRAb against TSHR, presenting a potential therapeutic approach for treating GD (Fig. 1). By elucidating the potential of CAR-T cell therapy in addressing autoimmune diseases like GD and its associated hyperthyroidism, we aim to contribute to the advancement of targeted and effective treatments for these complex conditions.

2. Materials and methods

2.1. Preparation of CHO-K1-TSHR recombinant cells

The extracellular domain of human TSHR (21-413aa) (UniProtKB-Q5ZPR3) was codon-optimized using GeneOptimizer from GeneArt and synthesized by GenScript USA. The synthesized fragment was then subcloned into the pcDNA3.1 vector. The recombinant expression vector was transfected into CHO-K1 cells (obtained from ATCC and maintained in our cell bank) using the LVTransm kit. Stable CHO-K1-TSHR recombinant cells constitutively expressing the TSHR on the cell membrane were selected by adding 400 μg/mL G418 to the culture medium for pressure culture.

2.2. Screening hybridomas

All animal experiments were approved by the Second Affiliated Hospital of Fujian Medical University Animal Care Committee. Five-week-old female BALB/C mice were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. The CHO-K1-TSHR recombinant cells were washed with physiological saline (Gbico, CAT# 14190–250) five times. Each mouse was injected with $5x10^6$ recombinant cells via the tail vein every 14 days. Ten days after the third injection, 100 μL of peripheral blood was collected via the tail vein, and the immune titer was detected by ELISA (Corning, CAT# 3365). Mice with the highest immune titer were selected, and $5x10^6$ recombinant cells were used for hybridoma fusion. The splenocytes of the mice were isolated three days after the final immunization. The fusion partner for hybridoma generation was SP2/0 cells. After three rounds of subcloning, the candidate hybridoma clones were successfully obtained. For ELISA assay, TSHR protein produced in 293F cells was pre-coated on a 96-well plate at 2 μg/mL in CBS buffer (pH 9.4). Mouse serum after immunization with TSHR protein and the culture medium of SP2/0 cells were used as positive and negative controls respectively.

2.3. Determination of the binding capability of TRAb with CHO-K1-TSHR

To determine the binding capability of TRAb with CHO-K1-TSHR cells, CHO-K1 cells were used as control cells. The TRAb derived from three subclone hybridomas were incubated with stable CHO-K1-TSHR recombinant cells. Each sample consisted of 0.5 μg of mouse monoclonal antibody incubated with $1x10^6$ stable CHO-K1-TSHR recombinant cells, followed by staining with a PE-conjugated secondary antibody(Sigma CAT# 301). The binding percentage of TRAb with CHO-K1-TSHR recombinant cells was then assessed using a flow cytometer (Thermo Attune NXT). This flow cytometry analysis allowed us to determine the specific binding affinity of TRAb subclones to CHO-K1-

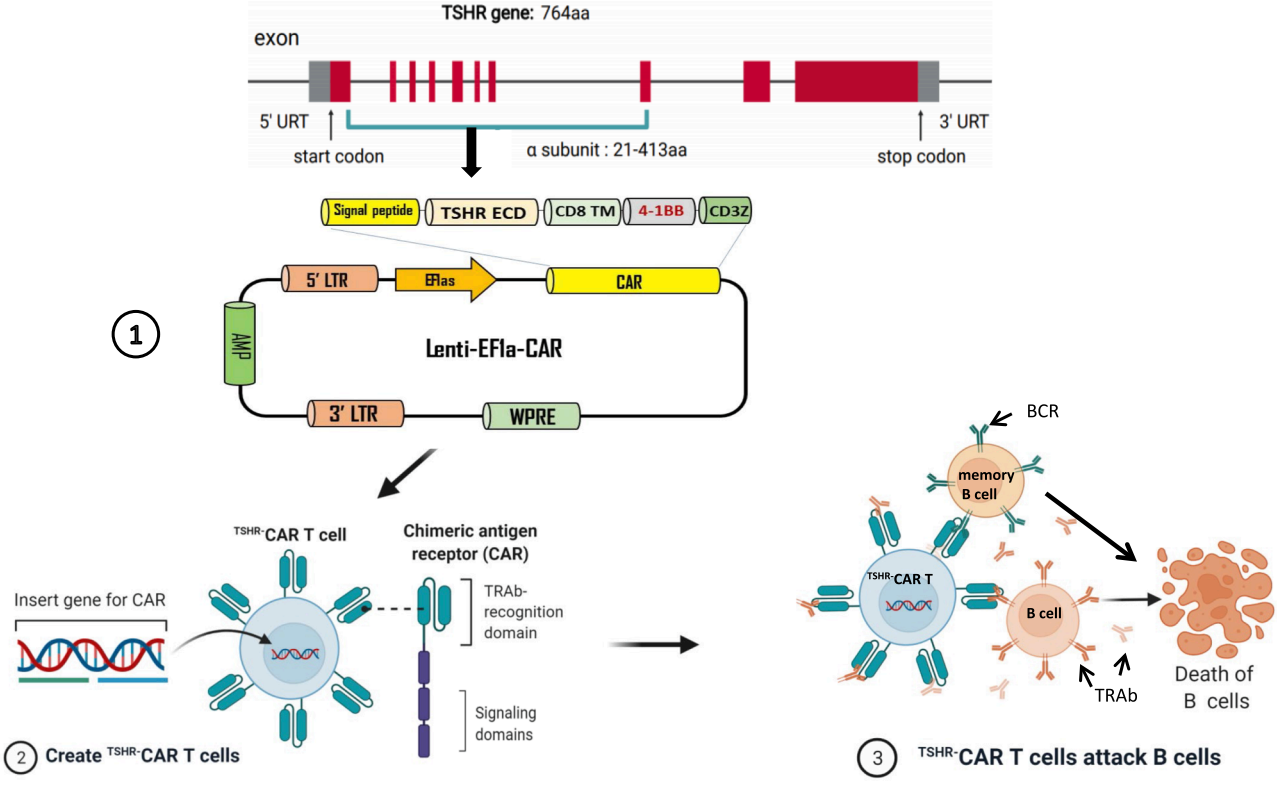

Fig. 1. Schematic illustrating the molecular process of TSHR-CAR-T cell creation and its specific recognition and cytotoxicity towards TRAb-producing auto-reactive B cells. Schematic diagram of the lentiviral vector is shown below. Signal peptide: CD8a; TSHR ECD: the first extracellular domain sequence of TSHR; CD8 TM: CD8 transmembrane region; 4-1BB: 4-1BB intracellular signal domain; CD3Z: CD3zeta intracellular signal domain.

TSHR cells and ascertain their potential as candidates for further investigation and therapeutic applications.

2.4. cAMP measurement

cAMP was measured using the cAMP immunoassay kit (Sigma CAT# 201-1KT). CHO-K1-TSHR cells were cultured for 2 days and then treated with TRAb clone 1–3 for 2, 5, 10, 30, 60, and 180 min, respectively. The culture medium was removed, and 0.1 M HCl was added. After mixing with acetylation reagent, the sample was loaded into a 96-well plate and incubated with cAMP antibody for one hour. Then, cAMP-HRP was added and incubated for another hour. After washing the plate three times with washing buffer, HRP chromogenic agent was added and incubated for 1 h. Finally, the reaction was terminated with 1 M HCl, and the color development was read at OD 450 nm using a plate reader. TRAb-(K1-18) was used as the negative control, which has the ability to bind to TSHR but lacks the ability to activate cAMP.

2.5. Construction of Hybridoma-Luciferase recombinant cells

Hybridoma clone 1 was chosen for the construction of Hybridoma-Luciferase recombinant cells. The coding region sequence information of Firefly luciferase was retrieved from the NCBI database. After gene synthesis, the synthesized fragment was subcloned into the lentivirus overexpression vector Lenti-CMV-puro using the BamHI-EcoRI (NEB, CAT# R3136S) restriction site. After verifying the insertion sequence by Sanger sequencing, lentivirus vectors were prepared. The sequence and fluorescent groups of the primers used are shown in Table 1. Puromycin (250 μL of 2X) was added to the culture medium, and the status of hybridoma cells was observed daily. The concentration of puromycin that completely killed all target cells on the fifth day was used for screening stable cells. Hybridoma cells were cultured in complete medium until the logarithmic growth period. After adding lentivirus for 24 h, the culture medium was replaced with medium containing puromycin and continuously cultured for 5 days until all uninfected target cells were killed. The remaining living cells were expanded, and luciferase activity was assessed in some of the cells. To investigate the luciferase activity of hybridoma-luciferase recombinant cells, ONE-Glo reagent was added into the 96-well plate containing the cells. The plate was incubated, and the luminescent signal was measured using an enzyme labeling instrument. The ONE-Glo assay allowed us to quantify the luciferase activity, providing essential information about the function of the hybridoma-luciferase recombinant cells.

2.6. Design and construction of TSHR-CAR lentivirus expression vector

The first extracellular domain sequence of the thyroid-stimulating hormone receptor (TSHR) (21-413aa) was selected based on the protein structure and sequence data from Uniprot. This selected sequence was then combined with a T cell activation signal domain to construct a lentiviral expression vector for the chimeric antigen receptor (CAR). The schematic diagram of the lentiviral vector is depicted in Fig. 1. The construction of the lentiviral expression vector involved codon optimization and synthesis of the full sequence at GenScript to ensure optimal expression. Subsequently, the synthesized CAR sequence was subcloned into the lentiviral vector. As a control, a CAR without the TSHR sequence was used. To generate the lentiviral supernatant, 293 T cells were co-transfected with the lentiviral CAR vector, along with gag/pol and VSVG components, using PEIMax. Following 48 and 72 h of transfection, we collected the lentivirus supernatant, passed it through a 0.45 μm filter, and concentrated it by subjecting it to ultracentrifugation at 19,000 RPM for 2 h. To ensure long-term preservation, we divided the lentivirus into aliquots and stored them at −80 °C. The lentivirus concentration was quantified using a qPCR method, as per the established protocol.

Table 1
The sequence and fluorescent groups of the primers.

Primer name	Sequence(5′-3)	Fluorescence labeling
WPRE F	GGCACTGACAATTCCGTGGT	
WPRE R	AGGGACGTAGCAGAAGGACG	
WPRE Probe	ACGTCCTTTCCATGGCTGCTCGC	5′-FAM-BHQ1-3′
ALB F	GCTGTCATCTCTTGTGGGCTGT	
ALB R	ACTCATGGGAGCTGCTGGTTC	
ALB Probe	CCTGTCATGCCCACACAAATCTCTCC	5′-FAM-BHQ1-3′

2.7. Preparation of TSHR-CAR-T cells

Human peripheral blood lymphocytes, adjusted to 1x106 cells/mL, were cultured in RPMI 1640 medium with 10% FBS and a mix of cytokines and antibodies, including IL-2 (10 ng/mL), IL-7 (5 ng/mL), IL-15 (100 ng/mL), Anti-CD3 (OKT3, 100 ng/mL), and Anti-CD28 (250 ng/mL) (Gbico, CAT# 61870044). Cells were then transduced with either TSHR-CAR or control CAR lentivirus. PBMCs were sourced from informed healthy volunteers.

The cells were cultured continuously for 48 h to prepare TSHR-CAR-T or control T cells. LVtransm transfection reagent (iCarTab, Cat# LVTran100) was used for lentivirus transfection.The surface expression of chimeric receptor on T cells was determined by flow cytometer after incubation with a fluorescence-labled antibody against TSHR. The percentage of chimeric receptor positive T cells is approximately 30%. The T cells engineered with a CAR lacking the TSHR sequence served as the control CAR-T.

2.8. In vitro co-culture of TSHR-CAR-T cells and Hybridoma-Luciferase cells

TRAb-secreting Hybridoma-Luciferase recombinant cells were employed as target cells, while TSHR-CAR-T cells served as effector cells and were cultured in serum-free 1640 medium. After gently washing the cells with sterile PBS, TSHR-CAR-T cells were added at various effector-target (E/T) ratios, ranging from 1:1 to 10:1. Human peripheral blood T cells were used as controls. Following a 6-hour co-culture, the supernatant was collected for INF-γ or IL-2 detection (methods referenced our previous article [2]). The plate was subsequently removed from the incubator, and the target cells were lysed using a lytic solution. After centrifugation at 1200xg at room temperature for 5 min, ONE-Glo detection reagent (ONE-Glo™ Luciferase Assay Substrate CAT# E606A) was added, and the luciferase value was measured using a luminometer.

2.9. Construction of mice GD model

The GD model was constructed by injecting B-NDG male mice with TRAb-producing (clone 1) Hybridoma-Luciferase recombinant cells, which secrete TRAb to stimulate thyroid function. Approximately $2x10^6$ cells were injected into each mouse via the tail vein. Seven days after the hybridoma cell implantation, the thyroid function (FT3, FT4) and TRAb were detected by electrochemiluminescence (Roche Diagnostics GmbH CAT# 03051986190–11731297122) to confirm the mice GD model.

2.10. In vivo assessment of TSHR-CAR-T cells on Hybridoma-Luciferase cells

Ten B-NDG male mice, 6 weeks old and weighing between 16 g and 22 g, were purchased from Beijing Biocytogen Co., Ltd. These mice were highly immunodeficient and capable of supporting human immune cell transplantation. After the mice were injected with TRAb-producing Hybridoma-Luciferase recombinant cells 3 days, they were then injected with either TSHR-CAR-T cells or control CAR-T cells, respectively. The activity, food intake, drinking water, and weight changes of the

mice were routinely monitored. Imaging was performed every 5–7 days to monitor the growth of hybridoma cells in vivo. Six days after the hybridoma recombinant cells implantation, TSHR-CAR T cells were delivered at a dose of $1x10^6$ per mouse.

2.11. Off-Target toxicity assessment

According to the standard protocol provided by the ELISA kit manufacturer, the off-target toxicity assessment involved the following procedures:

In vitro co-culture of TSHR-CAR-T cells with target cells included hybridomas secreting TRAb and primary mouse pituitary cells (ATCC, CAT# C39769). The cells were washed with sterile PBS, and TSHR-CAR-T cells were added at an effect-target ratio of 1:1 for co-culture lasting 6 h. The supernatant was then collected for cytokine detection using enzyme-linked immunosorbent assay (ELISA) (CAT# 6340).In vivo assessment of TSHR-CAR-T treatment on the GD model involved the detection of luteinizing hormone (LH) and testosterone levels using ELISA. The ELISA procedure followed the standard protocols provided by Shanghai Boke Biotechnology Co., Ltd., China (CAT# BKE6354).

All reagents and cell culture materials were sourced from Thermo, unless otherwise specified. These standardized procedures were conducted to evaluate the specificity and safety of TSHR-CAR-T cell therapy, both in vitro and in the GD model in vivo, by examining potential off-target effects.

2.12. Statistical analysis

SPSS 21.0 software was used for statistical analysis. Significance was determined by Student *t*-test or ANOVA. Results were expressed as the mean ± S.D. All reported p-values were two-tailed, and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Functional characterization of TRAb subclones in CHO-K1-TSHR cells

As TRAb is comprised of monoclonal antibodies recognizing distinct epitopes of TSHR, each subclone may exhibit specific pathophysiological functions. In this study, we observed that TRAb from different subclones had varying effects on cAMP levels in CHO-K1-TSHR cells.

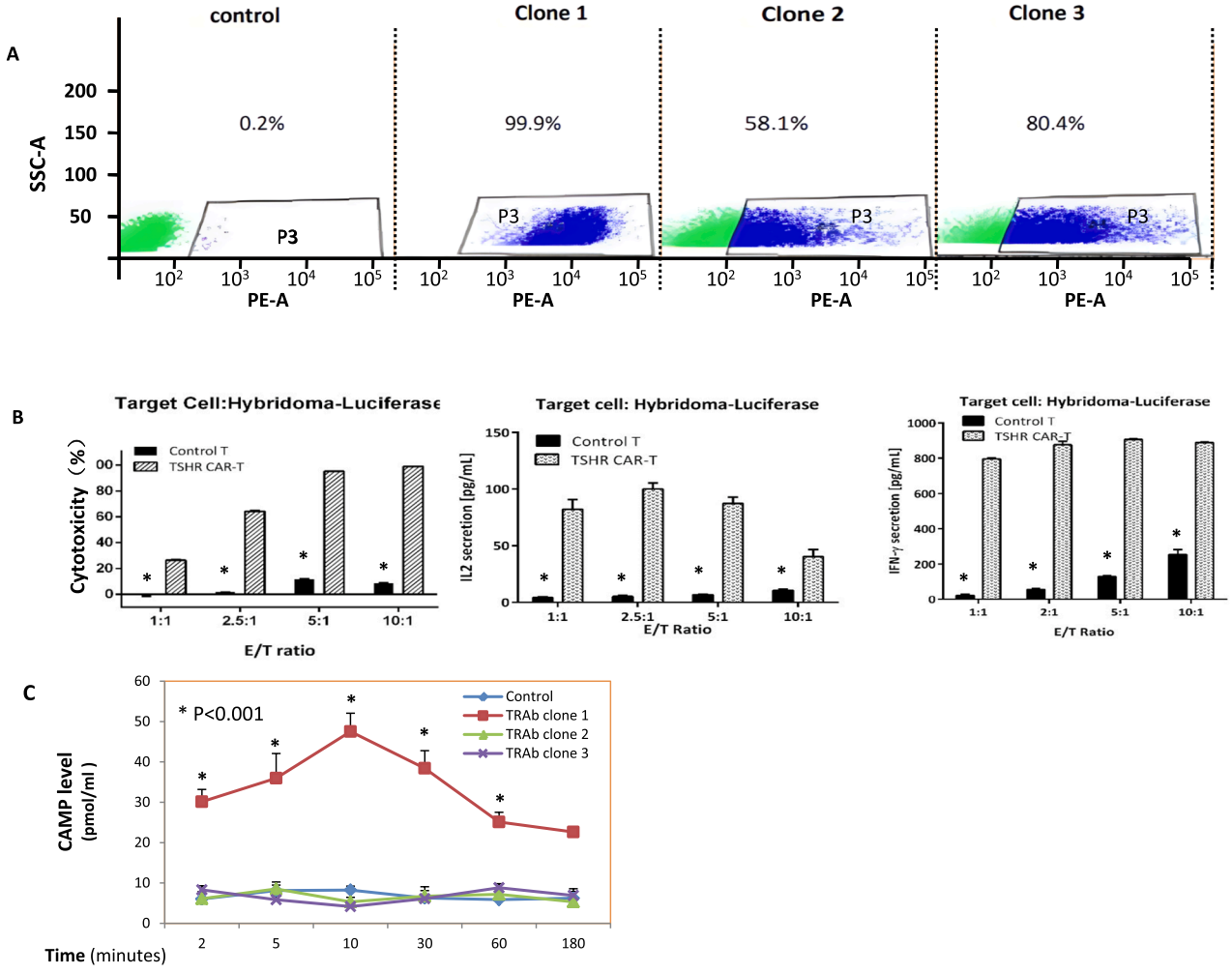

Fig. 2. TSHR-CAR-T cells demonstrated specific and potent cytotoxicity in vitro. (A) Representative flow cytometry analysis showing the binding rate of TRAb clone 1–3 to CHO-K1-TSHR cells.CHO-K1-TSHR recombinant cells were subjected to staining with TRAb from three different subclone hybridomas along with PE-conjugated secondary antibody. Flow cytometry analysis demonstrated that TRAb derived from subclone 1, subclone 2, and subclone 3 exhibited a remarkably high binding efficiency of 58.1–99.9% to the TSHR expressed on the cell membrane of CHO-K1-TSHR recombinant cells. In contrast, the control cells, CHO-K1 cells, showed a much lower binding rate, approximately 0.2%. (B) Specific cytotoxicity was evaluated by luciferase-based killing assay, and IL-2 and IFN-γ production by TSHR-CAR-T cells was measured by ELISA from co-culture supernatant at indicated effector to target (E/T) ratio. Statistical significance was determined using two-tailed Student's T test, *P < 0.001. (C) Treatment with TRAb clone 1 resulted in increased cAMP levels in CHO-TSHR cells, while clone 2 and clone 3 showed no significant changes. Biologically independent replicates (n = 3, P < 0.01). Data are presented as mean ± SEM. (Some error bars are too small to be visible).

Specifically, only TRAb from clone 1 significantly increased cAMP levels from 2 to 180 min, While TRAb from clone 2 and clone 3 did not elicit any effects on cAMP levels, the control group also showed no alterations in cAMP levels (Fig. 2C). These led us to select hybridoma subclone 1 for further investigation due to its distinct ability to induce increased cAMP levels through TSHR activation, resembling the pathophysiology of GD.

3.2. The effect of TRAb binding to CHO-K1-TSHR recombinant cells

CHO-K1-TSHR recombinant cells were subjected to staining with TRAb from three different subclone hybridomas along with PE-conjugated secondary antibody. Flow cytometry analysis demonstrated that TRAb derived from subclone 1, subclone 2, and subclone 3 exhibited a remarkably high binding efficiency of 58.1–99.9% to the TSHR expressed on the cell membrane of CHO-K1-TSHR recombinant cells. In contrast, the control cells and CHO-K1 cells, were showed a much lower binding rate, approximately 0.2% (Fig. 2A).

3.3. In vitro evaluation of TSHR-CAR-T cell effects on hybridomas recombinant cells

In the in vitro co-culture system, TSHR-CAR-T cells were effectively activated by target hybridomas, leading to a substantial secretion of IL-2 and IFN-γ cytokines. The levels of IL-2 and IFN-γ were significantly higher in the TSHR-CAR-T groups compared to the control T groups across different effector/target (E/T) ratios (TSHR-CAR-T/hybridoma 1:1, 2.5:1, 5:1, 10:1) ($n = 3$, $p < 0.001$)(Fig. 2B).

3.4. In vivo evaluation of TSHR-CAR-T cell effects on hybridomas recombinant cells

B-NDG mice were initially injected with hybridomas, followed by infusion of either TSHR-CAR-T cells or control T cells at day 6. In vivo imaging was performed regularly to monitor hybridoma growth and mice survival. The results revealed that in the control T cell group, hybridoma cells proliferated rapidly, leading to the untimely death of the mice. However, the infusion of TSHR-CAR-T cells effectively inhibited hybridoma proliferation and significantly prolonged the life cycle of the mice. Furthermore, mice in the TSHR-CAR-T group exhibited no significant weight loss, maintaining a stable body weight throughout the study period (20 ± 1.2 g vs. 13 ± 1.5 g on day 25th) (Fig. 3ABC).

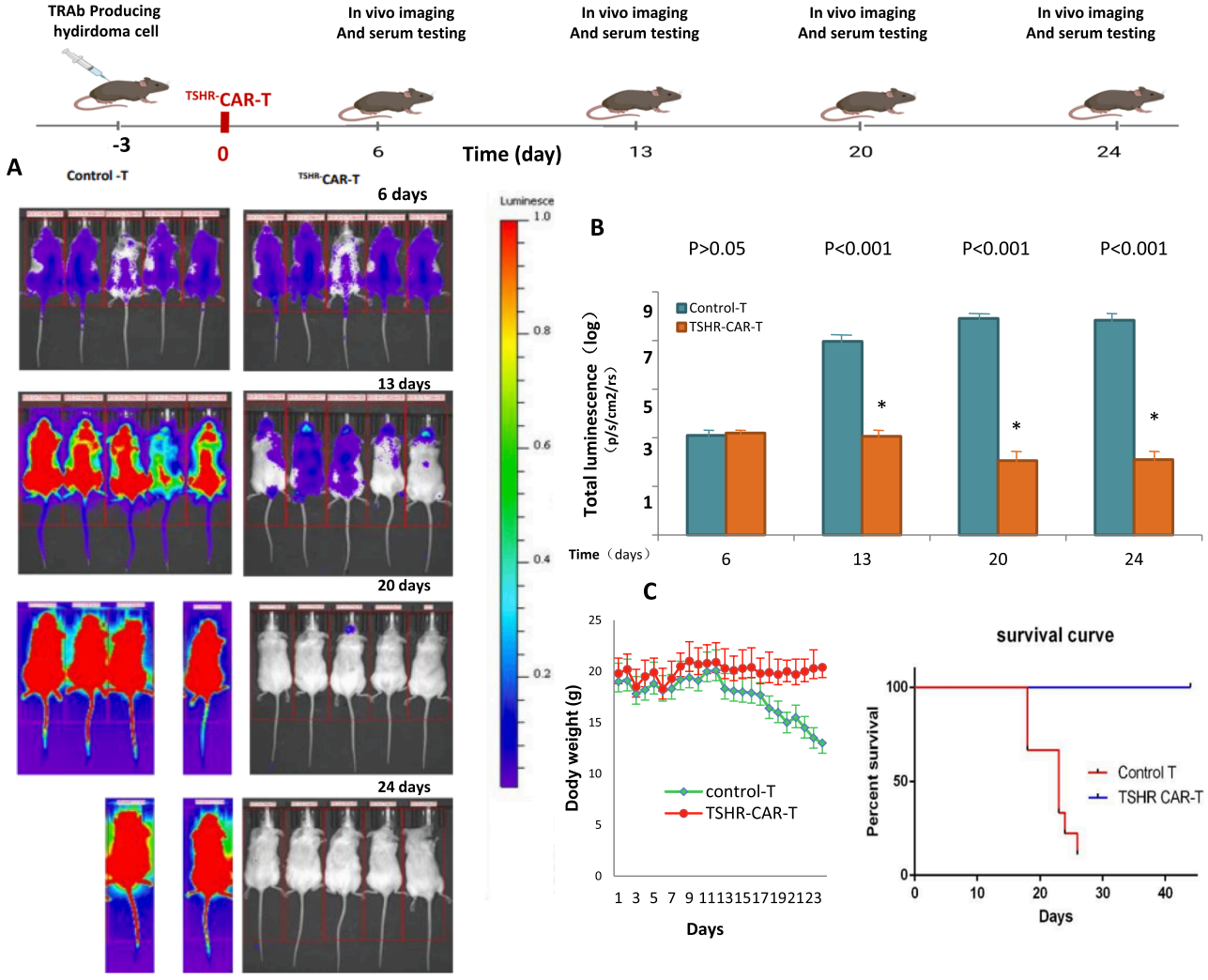

Fig. 3. TSHR-CAR-T cells specifically targeted and exhibited cytotoxicity to TRAb-producing B lymphocytes in vivo. B-NDG mice were injected with flyluciferaseTRAb-producing hybridomas, followed by either TSHR-CAR-T cell or control T cell injection at day 6, and in vivo imaging was conducted every5-7 days. (A) Serial quantification of hybridoma burden through bioluminescence imaging. (B) Bioluminescence imaging quantification of TSHR-CAR-T and control T cell groups at different time points, showing rapid hybridoma proliferation in the control T cell group. Symbols represent individual mice. (C) Significant decrease in body weight and survival rate in the control T cell group. Biologically independent mice ($n = 5$, $P < 0.01$) in two separate cohorts. Data are shown as mean ± SEM.

3.5. Therapeutic effect of TSHR-CAR-T cells in a Graves' disease model

After twenty days of intravenous injection of TSHR-CAR-T cells, the TSHR-CAR-T group showed a remarkable reversal of hyperthyroidism, as evidenced by the return of FT3, FT4, and TRAb levels to normal values (FT3: 23.2 ± 1.4 pmol/L vs. 3.4 ± 0.4 pmol/L, FT4: 65.4 ± 3.2 pmol/L vs. 38.1 ± 3.4 pmol/L, TRAb: 170 ± 16.2 mIU/ml vs. 1.71 ± 0.58 mIU/ml, n = 5, P < 0.001). Additionally, a significant reduction in thyroid gland volume was observed in the TSHR-CAR-T group. In contrast, the control group exhibited persistent elevation of FT3, FT4, and TRAb levels, along with the characteristic development of goiter (Fig. 4AB).

3.6. Off-target toxicity of TSHR-CAR-T cells in vitro and in vivo

To assess the safety of TSHR-CAR-T cells, we examined potential off-target effects on pituitary-expressed hormones, including thyroid stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are expressed in pituitary cells, can physiologically bind to TSHR. We conducted experiments using primary pituitary cells and TRAb-secreting hybridoma recombinant cells. TSHR-CAR-T cells exhibited specific cytolysis and secretion of IL2 and IFN-γ in response to TRAb-secreting hybridoma recombinant cells, indicating their specific targeting ability. Importantly, TSHR-CAR-T cells did not exhibit any response to pituitary cells, demonstrating the absence of off-target effects on these non-pathogenic cells. The levels of IFN-γ in the TRAb-secreting hybridoma recombinant cell group were significantly higher than in the pituitary cell group (81 ± 5.4 mg/ml vs 2.5 ± 0.21 mg/ml, 1008 ± 25.2 mg/ml vs 18.21 ± 1.24 mg/ml, n = 3, P < 0.001) (Fig. 4D).Furthermore, to assess the potential hormonal disruptions, we measured Luteinizing Hormone (LH) and Testosterone levels in the GD model treated with TSHR-CAR-T or control T cells in vivo. The results showed that LH and Testosterone levels were not noticeably altered in the TSHR-CAR-T group compared to the control T group (Fig. 4C). This finding indicates that TSHR-CAR-T cell administration did not cause any significant hormonal imbalances.

4. Discussion

Graves' disease (GD) is primarily caused by pathogenic auto-antibodies known as thyroid-stimulating immunoglobulins (TRAb), which are produced by auto-reactive B lymphocytes [24–26]. These TRAb antibodies stimulate the thyroid-stimulating hormone receptor (TSHR), resulting in the hyperthyroid symptoms observed in GD. TSHR is a G-protein-coupled receptor with four extracellular domains, and it has been demonstrated to specifically bind pathogenic auto-antibodies to the N-terminus of the first extracellular domain (21-413aa) [27–30].

In this study, we aimed to explore a novel approach for treating GD by utilizing chimeric antigen receptor T (CAR-T) cells specifically

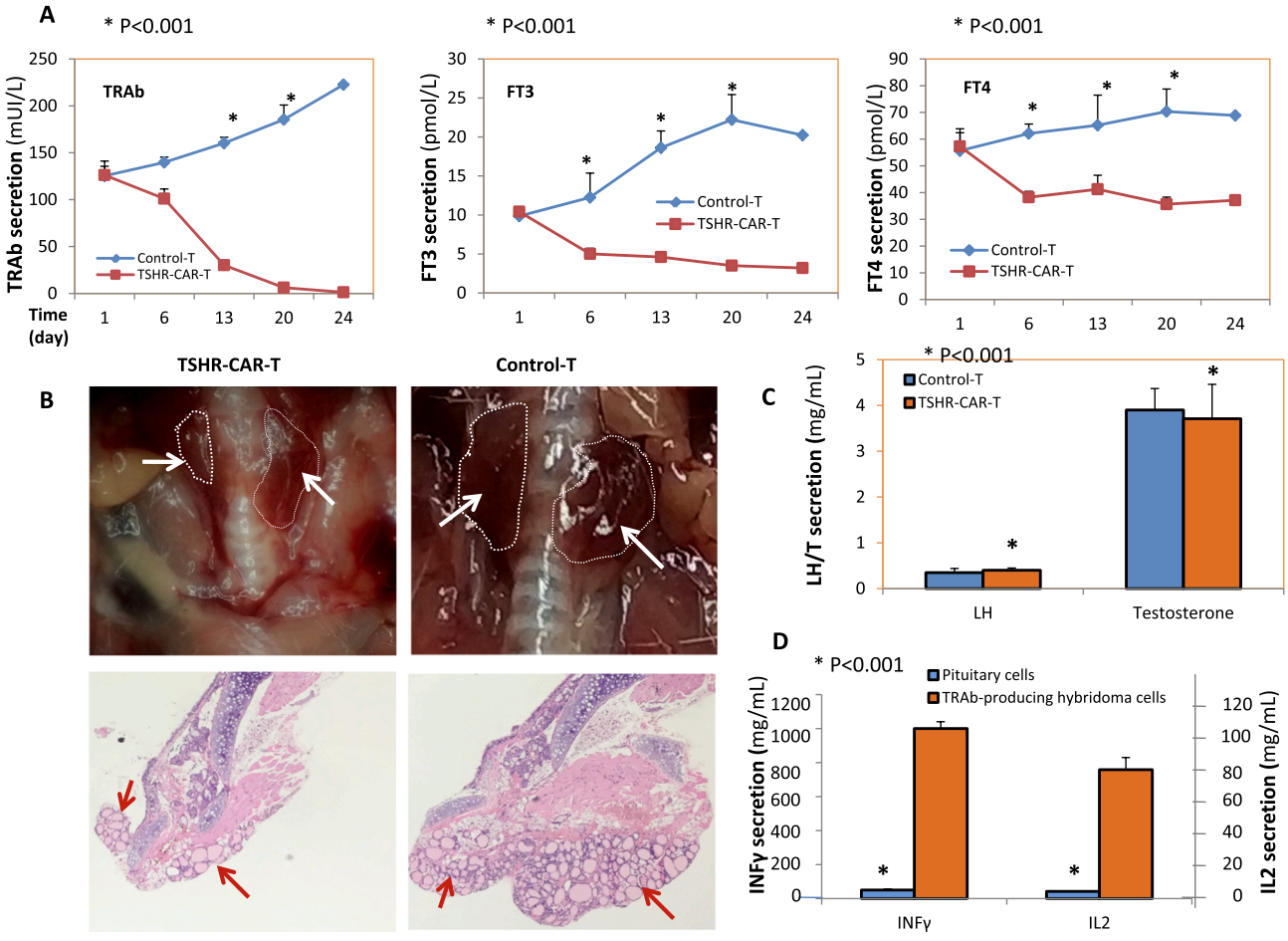

Fig. 4. TSHR-CAR-T therapy group restored thyroid function and did not exhibit valuable off-target toxicity in vivo. (A) After twenty days, FT3, FT4, and TRAb levels returned to normal in the TSHR-CAR-T group. (B) Significant improvement in thyroid goiter observed in the TSHR-CAR-T group (The arrow indicates thyroid tissue, and the dashed line represents the size of the thyroid). It can be observed that the thyroid size in the TSHR-CAR-T group is normal, while the control group shows significant enlargement of the thyroid. (C) ELISA analysis of LH and Testosterone in vivo. LH and Testosterone levels were not noticeably altered in the TSHR-CAR-T group compared to the control T group. (D) Pituitary cells or TRAb-producing hybridomas were incubated with TSHR-CAR-T cells for 6 h. Levels of IL-2 and IFN-γ in the TRAb-producing hybridomas group were significantly higher compared to the pituitary cells group. The results suggest TSHR-CAR-T cells do not exhibit specific cytolysis towards pituitary target cells. Biologically independent replicates (n = 3, P < 0.01). Data are shown as mean ± SEM.

designed to target TRAb-producing cells. Unlike conventional CAR-T cell therapy that employs single-chain variable regions (ScFv) as the antigen recognition domain, we designed the CAR-T cell to target the antigen fragment of pathogenic TRAb, specifically the first extracellular domain (21-413aa) of TSHR, fused with the CD8 transmembrane region, 4-1BB, and CD3zeta intracellular signal domain (Fig. 1).

To validate the specific recognition and binding capability of TSHR-CAR to TRAb, we employed CHO-K1-TSHR recombinant cells, which express the extracellular domain of TSHR on their cell membrane, and co-cultured them with TRAb derived from recombinant hybridomas. Through meticulous flow cytometry analysis, we successfully confirmed that TSHR on CHO-K1-TSHR recombinant cells exhibits a remarkably high affinity for specific binding to TRAb, particularly TRAb clone 1–3 (with binding rates ranging from 58.1% to 99.9%), in stark contrast to the control group (with a binding rate of 0.2%) (Fig. 2A).

Building upon the initial validation of recombinant TSHR's binding capability with diverse TRAb subclones, our study delved deeper into investigating the pathological and physiological functions of these TRAb subclones on CHO-K1-TSHR cells. As expected, we found that activating TRAb engagement with TSHR triggered intracellular signaling and resulted in elevated cAMP levels [31–33], whereas inhibitory antibodies did not elicit such responses. Furthermore, our research highlighted that one specific TRAb subclone (clone 1) demonstrated activating properties, effectively inducing intracellular cAMP elevation. These valuable insights shed light on the intricate pathogenesis of Graves' disease and offer promising prospects for targeted therapies in the future(Fig. 2C).

In the next step, we evaluated the killing ability of TSHR-CAR-T cells against TRAb-secreting cells at different effector/target ratios in an in vitro co-culture system. TSHR-CAR-T cells demonstrated specific cytolysis on target cells and exhibited a dose-dependent promotion of cytokine secretion, including elevated levels of IL-2 and IFN-γ, consistent with findings in other antibody-mediated autoimmune diseases [9]. Notably, TSHR-CAR-T cells exhibited robust cytotoxicity against clone 1 hybridomas, as evidenced by significant secretion of IL-2 and IFN-γ cytokines, while minimal cytokine secretion was observed in the control T cell group (Fig. 2B) [3,4]. These results emphasize the potent cytotoxic effect of TSHR-CAR-T cells against clone 1 hybridomas and further corroborate the specificity and functionality of TSHR-CAR-T cells in targeting TSHR-expressing hybridomas.In this study, the focus was solely on hybridoma clone 1 due to its ability to produceTRAb with the function of activating cAMP, leading to the development of hyperthyroidism similar to Graves' disease [34,35]. Therefore, other hybridoma clones, namely clone 2 and clone 3, were not investigated.However, it's better to set up the animal model using polyclonal hybridoma cells.

Moving to in vivo experiments, we utilized a severe immunodeficient B-NDG mouse model due to the inability of human CAR-T cells to survive in immunocompetent mice. This mouse strain allowed us to establish a model of Graves' disease (GD) by inducing the growth of clone 1 hybridoma cells. Simultaneously, we could engraft human CAR-T cells into the mice to evaluate the therapeutic efficacy of this approach. Using bioluminescence imaging, we quantified the hybridoma burden in vivo and observed that the aggressive proliferation of hybridomas led to weight loss and mortality in the mice. However, treatment with TSHR-CAR-T cells demonstrated specific cytolysis of hybridomas, resulting in reduced weight loss, prolonged survival, and decreased TRAb levels in the GD model mice [36–39]. Remarkably, mice treated with TSHR-CAR-T cells maintained stable body weight throughout the study period, highlighting the potential of TSHR-CAR-T cells as a promising treatment strategy for Graves' disease (Fig. 3ABC) [1,2]. We utilized a murine model and quantified hybridoma burden through bioluminescence imaging. Given the aggressive proliferation of hybridomas, they often cause weight loss and mortality in mice. As anticipated, TSHR-CAR-T cells exhibited specific cytolysis and effectively depleted hybridomas, leading to reduced weight loss, prolonged survival, and decreased TRAb levels in the GD model mice. Furthermore, mice in the TSHR-CAR-T group maintained stable body weight throughout the study period, further supporting the potential of TSHR-CAR-T cells as a treatment strategy for Graves' disease (Fig. 3ABC) [1,2].

These findings not only enhance our understanding of TRAb-related pathogenesis but also open up new avenues for further research and therapeutic applications in the treatment of Graves' disease. However, it is noteworthy to consider that antibodies present in the serum might bind to TSHR, potentially blocking the CAR receptor on T cells and compromising its functionality. This aspect warrants further investigation to optimize the clinical application of TSHR-CAR-T cells in patients with Graves' disease and other autoimmune disorders. Nonetheless, the promising in vivo and in vitro results suggest that TSHR-CAR-T cell therapy holds great promise in the field of precision medicine for autoimmune diseases, offering hope for improved management and potential cures in the future [1,2].

Furthermore, our study confirmed that treatment with TRAb clone 1 led to increased cAMP levels in thyroid cells, while clone 2 and clone 3 did not show significant changes (Fig. 2C) [5,6]. Based on these findings, we established the GD model using TRAb clone 1-producing hybridomas, which exhibited characteristic features of GD, including elevated FT3, FT4, and TRAb levels, goiter, and body weight loss [7,8]. Twenty days after intravenous administration of TSHR-CAR-T cells, we observed a successful reversal of hyperthyroidism, with FT3, FT4, and TRAb levels returning to normal values, and a significant improvement in thyroid goiter (Fig. 4AB) [9,10]. In contrast, the control T group showed persistent elevation of FT3, FT4, and TRAb levels, along with characteristic goiter development. These results demonstrate the successful therapeutic effect of TSHR-CAR-T cells in ameliorating hyperthyroidism and reversing GD-related abnormalities in the murine model [11,12].

To ensure the safety of TSHR-CAR-T cells, we evaluated potential off-target effects. Thyroid stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are expressed in pituitary cells, can physiologically bind to TSHR [40–42]. As expected, TSHR-CAR-T cells exhibited specific cytolysis and secretion of IL-2 and IFN-γ in response to TRAb-producing hybridomas, while showing no response to pituitary cells, indicating the absence of off-target effects on these non-pathogenic cells (Fig. 4D). We further examined hormone levels in the GD model treated with TSHR-CAR-T or control T cells in vivo. LH and Testosterone levels were not noticeably altered in the TSHR-CAR-T group compared to the control T group (Fig. 4C), demonstrating that TSHR-CAR-T cell administration did not cause any significant hormonal imbalances.

Considering the physiological relation between TSHR and TSH, as well as the structural similarities and affinity of LH and TSH to TSHR, there was a possibility of off-target effects impacting LH secretion and testosterone production. However, the absence of alterations in LH and Testosterone levels in the TSHR-CAR-T group supports the specificity and safety of the therapy, indicating that TSHR-CAR-T cells selectively target GD-associated TRAb-secreting cells without causing detrimental effects on normal pituitary function. These results underscore the promising potential of TSHR-CAR-T cell therapy as a targeted and safe approach for the treatment of Graves' disease. Based on our findings, we have reached the conclusion that TSHR-CAR-T cells demonstrate high specificity and low off-target toxicity, as demonstrated through their performance on hybridomas and primary pituitary cells. While the TRAb-secreting hybridoma cells can partially represent the autoantibody-secreting B lymphocytes, further investigations are warranted to explore their impact on recognizing TSHR-specific memory B cells and long-lived plasma cells, which play a crucial role in GD relapse. Additionally, more research is needed to evaluate potential off-target effects beyond pituitary cells.

In conclusion, our study provides compelling evidence for the potential of TSHR-CAR-T cells as a targeted treatment for GD. Through specific targeting and elimination of TRAb-producing hybridomas and reducing TRAb levels, TSHR-CAR-T cells demonstrated high specificity and low off-target toxicity, which are important characteristics for a successful immunotherapy (Fig. 1). The observed potent therapeutic

efficacy of TSHR-CAR-T cells in suppressing hybridoma growth, improving overall survival, and ameliorating hyperthyroidism further supports their potential as a promising immunotherapy for Graves' disease. The low off-target toxicity demonstrated by TSHR-CAR-T cells might be related to the subtle structural differences between the TSHR extracellular domain used in constructing the TSHR-CAR-T and the natural TSHR. This distinction thereby enhances the safety of this therapeutic approach.

This study lays a solid foundation for the development of TSHR-CAR-T cell therapy and its potential clinical applications for Graves' disease and related conditions. The encouraging results warrant further research and larger preclinical studies to validate these findings and pave the way for potential clinical translation. By addressing the unmet needs in the treatment of GD, TSHR-CAR-T cell therapy holds great promise as an innovative and effective therapeutic strategy for GD and potentially other autoimmune diseases.

5. Ethics statement

This study was conducted in accordance with the guidelines and recommendations of the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University. The protocol was approved by the ethics committee of the Second Affiliated Hospital of Fujian Medical University.

Author Contributions

HH conceived the project and designed the experiments. HD, ZJ, and LC performed the experiments. XB interpreted the data. XY and HC wrote and edited the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

[1] M. Mirzaee Godarzee, B. Mahmud Hussen, E. Razmara, B. Hakak-Zargar, F. Mohajerani, H. Dabiri, M. Fatih Rasul, M.H. Ghazimoradi, S. Babashah, M. Sadeghizadeh, Strategies to overcome the side effects of chimeric antigen receptor T cell therapy, Ann. N. Y. Acad. Sci. 1510 (1) (2022) 18–35.

[2] H. Duan, H. Huang, G. Jing, An Antibody Fab Fragment-based Chimeric Antigen Receptor Could Efficiently Eliminate Human Thyroid Cancer Cells, J. Cancer 10 (8) (2019) 1890–1895.

[3] H. Dai, W. Zhang, X. Li, Q. Han, Y. Guo, Y. Zhang, Y. Wang, C. Wang, F. Shi, Y. Zhang, M. Chen, K. Feng, Q. Wang, H. Zhu, X. Fu, S. Li, W. Han, Tolerance and efficacy of autologous or donor-derived T cells expressing CD19 chimeric antigen receptors in adult B-ALL with extramedullary leukemia, Oncoimmunology 4 (11) (2015) e1027469.

[4] Z. Yan, J. Cao, H. Cheng, J. Qiao, H. Zhang, Y. Wang, M. Shi, J. Lan, X. Fei, L. Jin, G. Jing, W. Sang, F. Zhu, W. Chen, Q. Wu, Y. Yao, G. Wang, J. Zhao, J. Zheng, Z. Li, K. Xu, A combination of humanised anti-CD19 and anti-BCMA CAR T cells in patients with relapsed or refractory multiple myeloma: a single-arm, phase 2 trial, The Lancet. Haematology 6 (10) (2019) e521–e529.

[5] Chimeric Antigen Receptor-Modified T Cells in Chronic Lymphoid Leukemia; Chimeric Antigen Receptor-Modified T Cells for Acute Lymphoid Leukemia; Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia, The New England journal of medicine 374(10) (2016) 998.

[6] X. Jin, Y. Han, J.Q. Wang, L. Lu, CAR-T cell therapy: new hope for systemic lupus erythematosus patients, Cell. Mol. Immunol. 18 (12) (2021) 2581–2582.

[7] D. Mougiakakos, G. Kronke, S. Volkl, S. Kretschmann, M. Aigner, S. Kharboutli, S. Boltz, B. Manger, A. Mackensen, G. Schett, CD19-Targeted CAR T Cells in Refractory Systemic Lupus Erythematosus, N. Engl. J. Med. 385 (6) (2021) 567–569.

[8] L. Zhang, T. Sosinowski, A.R. Cox, J.R. Cepeda, N.S. Sekhar, S.M. Hartig, D. Miao, L. Yu, M. Pietropaolo, H.W. Davidson, Chimeric antigen receptor (CAR) T cells targeting a pathogenic MHC class II:peptide complex modulate the progression of autoimmune diabetes, Journal of autoimmunity 96 (2019) 50–58.

[9] C.T. Ellebrecht, V.G. Bhoj, A. Nace, E.J. Choi, X. Mao, M.J. Cho, G. Di Zenzo, A. Lanzavecchia, J.T. Seykora, G. Cotsarelis, M.C. Milone, A.S. Payne, Reengineering chimeric antigen receptor T cells for targeted therapy of autoimmune disease, Science 353 (6295) (2016) 179–184.

[10] M. Su, C. Zhao, S. Luo, Therapeutic potential of chimeric antigen receptor based therapies in autoimmune diseases, Autoimmun. Rev. 21 (1) (2022) 102931.

[11] M. Tenspolde, K. Zimmermann, L.C. Weber, M. Hapke, M. Lieber, J. Dywicki, A. Frenzel, M. Hust, M. Galla, L.E. Buitrago-Molina, M.P. Manns, E. Jaeckel, M. Hardtke-Wolenski, Regulatory T cells engineered with a novel insulin-specific chimeric antigen receptor as a candidate immunotherapy for type 1 diabetes, J. Autoimmun. 103 (2019), 102289.

[12] R. Yoshihara, T. Komai, H. Shoda, K. Fujio, Elevation of cytomegalovirus antigenemia predicts serious infection and death in patients receiving immunosuppressive therapies for autoimmune diseases, International journal of rheumatic diseases 23 (11) (2020) 1534–1540.

[13] Z. Touma, D.D. Gladman, Current and future therapies for SLE: obstacles and recommendations for the development of novel treatments, Lupus science & medicine 4 (1) (2017) e000239.

[14] K.S. Jhaveri, I. Schlam, N.G. Holtzman, M. Peravali, P.K. Richardson, S. Dahiya, V. Malkovska, A.P. Rapoport, Safety and efficacy of CAR T cells in a patient with lymphoma and a coexisting autoimmune neuropathy, Blood Adv. 4 (23) (2020) 6019–6022.

[15] Y. Chen, J. Sun, H. Liu, G. Yin, Q. Xie, Immunotherapy Deriving from CAR-T Cell Treatment in Autoimmune Diseases, J. Immunol. Res. 2019 (2019) 5727516.

[16] A. Flemming, Autoimmune diseases: CAR-T cells take aim at autoimmunity, Nat. Rev. Drug Discov. 15 (9) (2016) 603.

[17] J.L. McGovern, G.P. Wright, H.J. Stauss, Engineering Specificity and Function of Therapeutic Regulatory T Cells, Front. Immunol. 8 (2017) 1517.

[18] Q. Zhang, W. Lu, C.L. Liang, Y. Chen, H. Liu, F. Qiu, Z. Dai, Chimeric Antigen Receptor (CAR) Treg: A Promising Approach to Inducing Immunological Tolerance, Front. Immunol. 9 (2018) 2359.

[19] H. Wardemann, S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, M.C. Nussenzweig, Predominant autoantibody production by early human B cell precursors, Science 301 (5638) (2003) 1374–1377.

[20] M. Radic, I. Neeli, T. Marion, Prospects for CAR T cell immunotherapy in autoimmune diseases: clues from Lupus, Expert Opin. Biol. Ther. 22 (4) (2022) 499–507.

[21] S.A. Beheshti, K. Shamsasenjan, M. Ahmadi, B. Abbasi, C.A.R. Treg, A new approach in the treatment of autoimmune diseases, Int. Immunopharmacol. 102 (2022), 108409.

[22] E. Elinav, N. Adam, T. Waks, Z. Eshhar, Amelioration of colitis by genetically engineered murine regulatory T cells redirected by antigen-specific chimeric receptor, Gastroenterology 136 (5) (2009) 1721–1731.

[23] R. Kansal, N. Richardson, I. Neeli, S. Khawaja, D. Chamberlain, M. Ghani, Q. U. Ghani, L. Balazs, S. Beranova-Giorgianni, F. Giorgianni, J.N. Kochenderfer, T. Marion, L.M. Albritton, M. Radic, Sustained B cell depletion by CD19-targeted CAR T cells is a highly effective treatment for murine lupus, Sci. Transl. Med. 11 (482) (2019).

[24] M. Rotondi, C. Virili, S. Pinto, F. Coperchini, L. Croce, N. Brusca, M. Centanni, L. Chiovato, The clinical phenotype of Graves' disease occurring as an isolated condition or in association with other autoimmune diseases, J. Endocrinol. Invest. 43 (2) (2020) 157–162.

[25] H.Y. Liu, Z.Y. Shi, D. Fan, S.X. Zhang, L.X. Wu, K.Y. Lu, S.Y. Yang, W.T. Li, J. F. Kang, C.H. Li, Z.H. Cheng, Y. Xue, Z.F. Wu, X.F. Li, S.J. Li, Absolute reduction in peripheral regulatory T cells in patients with Graves' disease and post-treatment recovery, Mol. Immunol. 144 (2022) 49–57.

[26] H. Huang, Y. Shi, B. Liang, H. Cai, Q. Cai, R. Lin, Optimal iodine supplementation during antithyroid drug therapy for Graves' disease is associated with lower recurrence rates than iodine restriction, Clin. Endocrinol. 88 (3) (2018) 473–478.

[27] H.P. Holthoff, K. Uhland, G.L. Kovacs, A. Reimann, K. Adler, C. Wenhart, M. Ungerer, Thyroid-stimulating hormone receptor (TSHR) fusion proteins in Graves' disease, J. Endocrinol. 246 (2) (2020) 135–147.

[28] C. Sugisawa, K. Abe, Y. Sunaga, M. Taniyama, T. Hasegawa, S. Narumi, Identification of compound heterozygous TSHR mutations (R109Q and R450H) in a patient with nonclassic TSH resistance and functional characterization of the mutant receptors, Clinical pediatric endocrinology : case reports and clinical investigations : official journal of the Japanese Society for Pediatric Endocrinology 27 (3) (2018) 123–130.

[29] R. Pujol-Borrell, M. Gimenez-Barcons, A. Marin-Sanchez, R. Colobran, Genetics of Graves' Disease: Special Focus on the Role of TSHR Gene, Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 47 (10) (2015) 753–766.

[30] J. Zhou, J. Chen, Y. Huang, X. Gao, C. Zhou, X. Meng, J. Sun, Signaling Dynamics of TSHR-Specific CAR-T Cells Revealed by FRET-Based Biosensors, Front. Cell Dev. Biol. 10 (2022), 845319.
[31] T.J. Smith, TSHR as a therapeutic target in Graves' disease, Expert Opin. Ther. Targets 21 (4) (2017) 427–432.
[32] S.D. Lytton, K.A. Ponto, M. Kanitz, N. Matheis, L.D. Kohn, G.J. Kahaly, A novel thyroid stimulating immunoglobulin bioassay is a functional indicator of activity and severity of Graves' orbitopathy, The Journal of clinical endocrinology and metabolism 95 (5) (2010) 2123–2131.
[33] A. Teniente-Serra, B. Soldevila, B. Quirant-Sanchez, M.A. Fernandez, A. Ester Condins, M. Puig-Domingo, R. Pujol-Borrell, E.M. Martinez-Caceres, Distinct pattern of peripheral lymphocyte subsets in Graves' disease with persistency of anti-TSHR autoantibodies, Autoimmunity 52 (5–6) (2019) 220–227.
[34] K.H. Usadel, J. Teuber, R. Paschke, M. Junker, U. Schwedes, Transplantation of human endocrine tissues to nude mice: a suitable in vivo model for the study of pathomechanisms involved in autoimmune thyroid diseases, Acta Endocrinol. Suppl. 281 (1987) 77–81.
[35] Y. Tang, X. Zhu, H. Feng, L. Zhu, S. Fu, B. Kong, X. Liu, An improved mouse model of Graves disease by once immunization with Ad-TSHR289, Endocr. J. 66 (9) (2019) 827–835.
[36] H. Li, T. Wang, The autoimmunity in Graves's disease, Front. Biosci. 18 (2) (2013) 782–787.
[37] L. Frommer, J. König, S. Chatzidou, G. Chionos, J. Längericht, G.J. Kahaly, Recurrence risk of autoimmune thyroid and endocrine diseases, Best practice & research. Clinical endocrinology & metabolism 37 (2) (2023) 101636.
[38] S. Kang, J. Kang, H. Shen, N. Wu, Advances in regulatory B cells in autoimmune thyroid diseases, Int. Immunopharmacol. 96 (2021), 107770.
[39] A. Schluter, M. Horstmann, S. Diaz-Cano, S. Plohn, K. Stahr, S. Mattheis, M. Oeverhaus, S. Lang, U. Flogel, U. Berchner-Pfannschmidt, A. Eckstein, J. P. Banga, Genetic immunization with mouse thyrotrophin hormone receptor plasmid breaks self-tolerance for a murine model of autoimmune thyroid disease and Graves' orbitopathy, Clin. Exp. Immunol. 191 (3) (2018) 255–267.
[40] C. Giuliani, D. Cerrone, N. Harii, M. Thornton, L.D. Kohn, N.M. Dagia, E. Fiore, I. Bucci, T. Chamblin, P. Vitti, F. Monaco, G. Napolitano, A TSHr-LH/CGr chimera that measures functional TSAb in Graves' disease, J. Clin. Endocrinol. Metab. 97 (7) (2012) E1106–E1115.
[41] C. Giuliani, D. Cerrone, N. Harii, M. Thornton, L.D. Kohn, N.M. Dagia, I. Bucci, M. Carpentieri, B. Di Nenno, A. Di Blasio, P. Vitti, F. Monaco, G. Napolitano, A TSHR-LH/CGR chimera that measures functional thyroid-stimulating autoantibodies (TSAb) can predict remission or recurrence in Graves' patients undergoing antithyroid drug (ATD) treatment, J. Clin. Endocrinol. Metab. 97 (7) (2012) E1080–E1087.
[42] L. Wide, K. Eriksson, Molecular size and charge as dimensions to identify and characterize circulating glycoforms of human FSH, LH and TSH, Upsala journal of medical sciences 122 (4) (2017) 217–223.