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Thyroid-stimulating hormone receptor (TSHR) fusion proteins in Graves' disease

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Abstract

Graves' disease is an autoimmune disorder, which is characterized by stimulatory antibodies targeting the human thyrotropin receptor (TSHR), resulting in hyperthyroidism and multiple organ damage. We systematically investigated monomeric and dimeric fusion proteins of the A subunit of TSHR for efficacy to bind to the monoclonal patient antibody M22, to interact with Graves' patient serum samples, and to impact on anti-TSHR antibody titers, hyperthyroidism, tachycardia and other *in vivo* read-outs in a long-term mouse model of Graves' disease induced by immunization with a recombinant adenovirus encoding TSHR A. Binding assays and functional measurements of TSHR-dependent cAMP formation showed binding of monomeric TSHR-His and dimeric TSHR-Fc to the anti-TSHR antibody M22 at low-effective concentrations (EC_{50} of 5.7 nmol/L and 8.6 nmol/L) and inhibition of the effects of this antibody at high efficiencies (IC_{50} values of 16–20 nmol/L). Both proteins also block the effects of polyclonal anti-TSHR antibodies occurring in Graves' patient sera with somewhat lower average efficiencies (mean IC_{50} values of 29 nmol/L and 68 nmol/L). However, *in vivo* characterization of epicutaneous patch administrations of TSHR-Fc at doses of 0.3 and 0.6 mg/kg body weight in a murine Graves' disease model did not result in any improvement of disease parameters. In conclusion, high affinity binding of TSHR-Fc to pathological anti-TSHR antibodies was not matched by efficacy to improve Graves' disease parameter in a long-term mouse model.

Key Words:

▶ thyroid
▶ Graves' disease
▶ autoimmunity
▶ peptides

Introduction

In patients with Graves' disease (GD), antibodies which target the thyrotropin-TSH receptor (TSHR) in the thyroid gland and other autoimmune markers are observed. This autoimmune condition results in hyperthyroidism (Weetman 2000, Smith & Hegedüs 2016), with an annual incidence of 20–80 per 100,000 persons throughout the world. If left untreated, Graves' leads to significantly increased morbidity and mortality (Ross 2011). Graves' orbitopathy afflicts about 50% of patients and is especially hard to treat (Bahn 2010).

Despite the central role for the TSH holoreceptor (TSHR) in increasing thyroid hormone synthesis and secretion after ligand binding, strong experimental evidence suggests that it is not the holoreceptor but the

A-subunit, a shed component of the extracellular domain, that is the primary immunogen in the induction and affinity maturation of pathologic stimulating anti-TSHR antibodies (Chen *et al.* 2003, Mizutori *et al.* 2009).

Preparations of the secreted TSHR ectodomain variant TSHR-289, essentially the TSHR A-subunit, contained two different forms, with reciprocally exclusive binding characteristics to anti-TSHR autoantibodies. The temperature-sensitive form (also termed 'immunologically active' by the group of Rapoport and colleagues) neutralized the TSHR binding, stimulatory activity of Graves' sera, whereas the temperature-insensitive form (also termed 'inactive') was not reactive with sera from Graves' patients (Chazenbalk *et al.* 1999, 2001, Schwarz-Lauer *et al.* 2002, Miller-Gallacher *et al.* 2019). In contrast, the temperature-insensitive form was bound by the murine monoclonal anti-TSHR antibody 3BD10 which did not bind to the temperature-sensitive A-subunit in the preparation (Chazenbalk *et al.* 1999, 2001, Schwarz-Lauer *et al.* 2002). The human Graves' patient-derived monoclonal anti-TSHR antibody M22 was shown to bind specifically to the temperature-sensitive TSHR A-subunit (Chazenbalk *et al.* 1999, Hamidi *et al.* 2013, Rapoport *et al.* 2015).

The temperature-sensitive A-subunit has been shown to be unstable at 37°C. At this temperature it transforms into the temperature-insensitive A-subunit within 1–7 h, depending on the presence of stabilizing agents in the medium (Chazenbalk *et al.* 1999, 2001, Schwarz-Lauer *et al.* 2002). In contrast, at ambient temperature and at 27°C, the temperature-sensitive A-subunit is stable. In previous studies, the binding specificity of 3BD10 to the temperature-insensitive TSHR A-subunit had been used to separate the two TSHR A-subunits after recombinant expression, that is, the temperature-insensitive form was removed from the preparation by affinity chromatography with 3BD10, leaving the isolated temperature-sensitive form.

The temperature sensitivity was assimilated in a protocol for efficient recombinant expression of a tagged protein of temperature-sensitive extracellular TSHR domain (aa 21–261) and alkaline phosphatase (used as a molecular probe to directly detect the binding of anti-TSHR antibodies from patient samples in a bridging ELISA) by secretion of the molecules for 24 h at 27°C (Frank *et al.* 2015). This manuscript describes that we reconfirmed this protocol and produced temperature-sensitive forms of the TSHR A-subunit by transferring the expression cell lines to 27°C.

We then wished to characterize temperature-sensitive forms of a His-tagged TSHR A domain, as well as of a Fc fusion protein of this TSHR domain, which expresses two of these domains per molecule, in several assays including Graves' disease patient blood samples *ex vivo*. In addition, we investigated the capacity of the Fc fusion protein to mitigate disease parameters in a long-term mouse model of the disease. Previously, we had described this long-term disease model for human Graves' disease with nine immunizations of recombinant adenovirus expressing the extracellular A subunit of the TSHR (Holthoff *et al.* 2015) to permanently boost antibody production in mice. The TSHR A domain variants were tested for their potency to impact on long-term disease parameters in TSHR-immunized mice, such as hyperthyroidism (T4 values), thyroid size and tachycardia. A pivotal previous study had shown that while pre-administration of TSHR A domain attenuated the induction of hyperthyroidism by subsequent immunization with the TSHR A domain adenovirus by diverting the antibody response, s.c. injection of TSHRA domain was ineffective when administered once after disease induction in a short-term model (Misharin *et al.* 2009, observation for 5 weeks after single therapy). Since i.v. administration of TSHR-Fc led to signs of allergy and slight anti-TSHR antibody titer elevations in the long-term mouse model (Holthoff *et al.* 2017), we wished to investigate repeated epicutaneous/transdermal application instead, as has been proposed in several studies on other autoimmune diseases such as multiple sclerosis in preclinical and clinical studies (Bynoe *et al.* 2003, Walczak *et al.* 2013, Wildner & Selmaj 2017). Antigen-specific approaches may target anti-TSHR antibodies directly, or respective specific B cell receptors, or epidermal Langerhans cells, which then typically present the antigen to further immune cells. In this study, we only chose TSHR-Fc for *in vivo* experiments, since their purification is easiest, and epicutaneous antigen administration presumably favors presentation of processed peptides by Langerhans cells to T cells, and the amino acid sequence of TSHR is the same in TSHR-Fc or TSHR-His.

Materials and methods

Human serum samples

Serum samples from 48 patients with suspected GD were taken at Flor Ferenc Hospital, Kistarcsa, Hungary. This study was approved by the Hungarian Ethics Committee at Budapest (Egeszsegügyi Tudomanyos Tanacs), which issued an independent ethics vote 7022/2016/EKU (0102/16) on February 24, 2016.

The principles of the Declaration of Helsinki were respected. Consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used.

All patients had clinical signs of hyperthyroidism. Mean subject age was 38.8±13.7 and the female/male proportion was 77% female, 23% male. Most patients with GD had been medically treated with antithyroid drugs.

Serum samples from 17 healthy volunteers served as controls.

Cloning, transfection, production, and purification of human TSHR-His

Generation of a CHO TSHR289 His cell line

TSHR-His consists of the first 289 amino acids of the human TSHR (extracellular TSHR A subunit) followed by a Factor Xa cleavage site and six histidines. The cDNA sequence adapted for hamster codon usage was produced synthetically by GeneArt (Life Technologies) and cloned into the plasmid vector pcDNA5/FRT via HindIII and XhoI. The resulting expression vector was transfected into Flp-In™-Chinese hamster ovary (CHO) cells (Life Technologies), together with the plasmid pOG44, providing site-directed recombination. After selection of a stably expressing clone in Ham's F12 supplemented with 10% fetal bovine serum and 600 µg/mL Hygromycin B, the clone was adapted to ProCHO5 medium (Lonza, #BE12-766Q) supplemented with 4 mM L-glutamine (Biochrom, Cambridge, UK; #K0283).

The resulting vector was called pcDNA5/FRT-TSHR-His and allows expression and secretion of TSHR-His into the culture medium of mammalian cells under the control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter and selection for stable clones with Hygromycin B after co-transfection with plasmid pOG44.

Expression and purification of temperature-sensitive and temperature-insensitive TSHR-His

For the expression of TSHR-His, CHO-TSHR289-His cells were grown in ProCHO5, 4 mM L-glutamine in flasks to submaximal density at 37°C and then centrifuged. For expression of the temperature-sensitive TSHR-His, the cell pellet was resuspended in one-third of the original volume of the medium and incubated for 24 h at 27°C. For the expression of temperature-insensitive TSHR-His, the cells were continuously grown at 37°C. The supernatants were cleared by centrifugation at 4000 *g* for 10 min and subsequent filtration with a 0.22 nm sterile filter (TPP, Trasadingen, Switzerland; #99722). The resulting

TSHR A subunit containing medium was immediately frozen and stored at −80°C until protein purification. The supernatants from the 37°C and 27°C cell cultures were used for the preparations of TSHR-His containing more temperature-insensitive and temperature-sensitive TSHR-His, respectively.

For protein purification, thawed CHO-TSHR289-His supernatants were diluted 1:4 in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4, and loaded on an equilibrated 1 mL HisTrap™ excel column (GE Healthcare 17-3712-05). After washing the column with 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4, TSHR-His was eluted with 4×0.5 mL 20 mM sodium phosphate, 0.333 M NaCl, 0.333 mM imidazole, pH 7.4. Protein content was determined by OD 280 measurement and the relevant fractions were dialysed (Slyde-A-Lyzer Dialysis Cassette, 10000 MWCO, Thermo Scientific # 66380) against PBS at 4°C for 16 h.

Expression and purification of the TSHR A subunit-Fc fusion protein

The fusion protein TSHR-Fc consisting of the first 289 amino acids of the human TSHR followed by a GGR linker and the Fc portion of human immunoglobulin G (IgG) 2 was produced based on what was previously described (Holthoff *et al.* 2017). However, in that study a predominantly temperature-sensitive form was produced in ProCHO5, 4 mM L-glutamine in flasks instead of spinner culture, and culture was performed at 27°C instead of 37°C.

Measurement of human anti-TSHR antibodies by competitive sandwich ELISA

All procedures were performed at room temperature (RT) and incubations were on a microtiter plate shaker. ELISA plates were coated with 100 µL/well well-characterized, high-affinity monoclonal anti TSHR antibody M22 (RSR Limited, Cardiff, UK) in coating solution for 1 h. The coated plates were washed three times with PBST (PBS, 0.1% Tween-20), blocked with 100 µL/well of blocking solution (PBST, 3% milk powder) for 1 h, and washed again. Either 6 µL/well serum sample+24 µL PBST or 30 µL standard solution (spiked with human serum received from third generation ELISA kit, RSR Limited), respectively, was mixed with 30 µL/well of 2 µg/mL temperature-sensitive TSHR-His or TSHR-Fc in a dilution plate and incubated for 0.5 h. Afterwards the different mixes were transferred to the blocked ELISA plates and incubated for 1 h. After washing with PBST, the ELISA plates were incubated for 1 h either (for TSHR-His) with

100 µL/well of the anti-His detection antibody labeled with POD (Bethyl, Montgomery, TX, USA; #A190-114P, 1:3000 dilution in PBST) or (for TSHR-Fc) with polyclonal anti-TSHR antibodies from Ad-TSHR-immunized mice followed by POD-labelled anti-mouse Fc Fab antibodies (Jackson #115-036-072). After washing, bound POD was detected by incubation with 100 µL/well of TMB substrate (Thermo Scientific, #34029) until a maximal optical density (OD) of about 1–2 was reached. Finally, the colorimetric reaction was stopped with 100 µL/well stopping solution (1 M H_2SO_4) and the OD determined at a wavelength of 450 nm with a reference wave length of 595 nm with in a plate reader. The binding of TSHR subunit A-His and TSHR-Fc to coated M22 was analyzed with the same procedure, however, with varying concentrations of protein and without pre-incubation with serum or anti-TSHR antibody.

Determination of stimulatory anti-TSHR antibodies with the cAMP assay

TSHR-stimulating antibodies in the serum of Graves' disease patients were analyzed by measuring cyclic AMP (cAMP) generation in CHO cells JP2626 expressing the human TSHR (kindly provided by Dr Gilbert Vassart, Brussels, Belgium). CHO cells were seeded into 96-well plates (30,000 cells per well) and incubated for 24 h in Dulbecco's modified Eagle medium (DMEM, Invitrogen Ltd) containing 2% fetal calf serum. Then, DMEM was removed and human serum was diluted 1:5 in 50 µL HBSS buffer (20 mM Hepes, 1.26 mM $CaCl_2$, 5.33 mM KCl, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 5.6 mM glucose, and 222 mM sucrose, pH 7.2) supplemented with 1.5% BSA and 0.5 mM isobutyl-1-methylxanthine (Sigma-Aldrich) and added to each well. After incubation for 2.5 h at 37°C the cAMP release in the medium was measured in duplicates by a competitive ELISA (#EMSCAMPL, Thermo Fisher Scientific).

Determination of anti TSHR antibodies with the third-generation assay

Human antibodies against TSHR were detected by a commercially available TSH receptor autoantibody third-generation enzyme immunoassay (# TRE/96/3A) provided by RSR Limited, in which the human Graves patient-derived M22 MAB and serum antibodies compete for binding sites on immobilized TSHR. The assay was performed using 30 µL (instead of 75 µL) human serum in at least duplicate determination according to the manufacturer's instructions.

Multimer analysis by sedimentation velocity ultracentrifugation

In order to compare the quarternary structure of stressed and unstressed TSHR-Fc, an aliquot of 1.6 mg/mL TSHR-Fc was stressed by incubating the protein in PBS at 37°C for 24 h. Stressed and unstressed TSHR-Fc were subjected to multimer analysis by sedimentation velocity ultracentrifugation which was carried out at Coriolis Pharma Research GmbH (Planegg, Germany). For SV-AUC measurements, samples were used at a concentration of 0.8 mg/mL (corresponding to –1 OD 280, 1.2 cm in the AUC cell). Each sample was analyzed in triplicate in a ProteomeLab XL-I analytical ultracentrifuge (Beckman-Coulter) at 20°C, by using an 8-hole An-50 Ti analytical rotor and 12-mm epon-charcoal double-sector centerpieces with sapphire windows.

Of each sample, 340 µL were loaded into the sample sector of the double-sector centerpiece, and 360 µL of PBS into the reference sector in order to distinguish sample from buffer menisci. Prior to the measurement, sample concentrations and absence of leaks were confirmed at 3000 rpm (1000 g), followed by at least 1 h of sample and rotor temperature equilibration with resting rotor. The evolution of sedimentation profiles at 35 k rpm (102,700 g) was recorded by using absorbance optics in intensity mode at a wavelength of 280 nm. Data analysis was performed with Sedfit (version 15.01b). Buffer density and viscosity values as well as the partial specific volume for the protein (based on the amino acid sequence) were estimated with UltraScan. By using Sedfit, SV-AUC raw data were pre-processed (meniscus position fixed, bottom position fit, and time-invariant and radially invariant noise correction) and analyzed by using the continuous c(s) distribution model (s range: 0–22 S) with Tikhonov maximum entropy regularization (confidence level: 0.95) for the assessment of continuous sedimentation coefficient c(s) distributions.

Studies in immunized mice

Recombinant adenovirus containing the DNA sequence of the first 289 amino acids of the human TSH receptor were used as described before (Holthoff *et al.* 2015). Female BALB/c mice were delivered from Charles River and were adapted for at least 1 week to start experiments at the age of 6 weeks. Animals were kept under standard housing

conditions (23±2°C, 55±10% RH) in groups of ten animals in GR1800DD cages (Tecniplast®). All animal experiments were approved by the local animal welfare authority and Ethics committee at the Regierung von Oberbayern (Government of Upper Bavaria) in Munich, Germany (no. 55.2-1-54-2531-25-12), and carried out in accordance to the World Medical Association (Declaration of Helsinki), and the European Commission guidelines (Directive 2010/63/EU). All guidelines for care of animals were respected.

Immunizations

Mice received 10^{10} plaque-forming units (pfu) of adenovirus carrying the A-subunit (AA 1–289) of the TSHR gene (Ad-TSHR289) or green fluorescent protein (Ad-GFP, immunized control). In addition, age-matched immunologically naïve mice which were not immunized and were not treated were studied for comparison as healthy control group. For immunization, mice were anaesthetized with isoflurane (introduction 5%, maintenance 1.5–2%) and placed on a heating pad. The adenovirus was injected into the left and right femoral muscles in a volume of 25 µL each.

Blood withdrawals

For blood withdrawal, mice were moved to a restrainer. 100 µL of blood was withdrawn out of the left or right tail vein with a 27G needle. Blood was centrifuged at 2400 *g* for 15 min at room temperature and serum was stored at –20°C. At the end of the study (before euthanasia), blood was withdrawn intracardiacally in deep anesthesia (170 mg/kg ketamine+17 mg/kg xylazine) with a 1 mL syringe and a 24G needle and treated as mentioned above.

The study protocol used three 3-weekly immunizations ('initiation'), followed by a maintenance phase with further regular 4-weekly boosts until the 9th immunization, with either Ad-TSHR or control Ad-GFP (green fluorescent protein), as described before (Holthoff *et al.* 2015). Strict inclusion and exclusion criteria were defined before starting the study: If T4 thyroxin values in Ad-TSHR289-immunized mice were at least 25% higher at week 11 compared to basal measurement at week 0, mice were included for further study. Consequently, 25% of immunized mice were excluded before randomization because they did not meet this threshold. Also, mean T4 values and basal heart rates of all groups had to be consistently altered. Remaining mice were randomly assigned to therapeutic groups, allocations were concealed, and veterinarians and all other staff who handled and analyzed the animals, were strictly blinded to the treatment groups.

Drug administrations

TSHR-Fc (0.3 or 0.6 mg/kg bw) or 0.9% NaCl vehicle control were given 1 week after the 4th immunization by epicutaneous patches over 24 h (Finn Chambers Aqua acquired from SmartPractice, Phoenix, AZ, USA, diameter: 8 mm), after shaving mouse backs on a 2.5×2.5 cm area. About 25 µL of drug or vehicle solution were applied in each Finn Chamber, and due to small variations in pipetting and body weight of mice, we estimate dosing error at max. ± 8%. This treatment was then repeated at 4-weekly intervals, as described before for i.v. peptide administrations (Holthoff *et al.* 2017). No animal was excluded after randomization.

Determination of heart rate, ECG, and organ analysis

When mice were subjected to anesthesia for immunization, their heart rate was monitored by an ECG (ECG amplifier module, Harvard Apparatus, Hugo Sachs Electronik, March, Germany) and recorded with a special software, which allows to determine the heart rate from the ECG reading (Haemodyn, Hugo Sachs Electronik). ECG was also performed in anesthesia before animals were killed for histological exam at the end of the study.

After killing, dissection and determination of the organ size of the thyroid glands was performed under a stereomicroscope, as described before (Holthoff *et al.* 2015).

Measurements in mouse sera

Total thyroxine (T4) was measured by using an immunoassay kit (#T4044T-100, Calbiotech Inc, Austin, CA, USA) in duplicate determination.

Anti-TSHR autoantibody titers and potency of antibodies to stimulate TSHR-dependent cAMP levels in test cells were determined before start of immunisation (basal value), 56 days after the first immunisation, 133 days after the first immunisation, and 189 days after the first immunisation, and at the end of experiment. To this aim, a '3rd generation assay' was used: antibodies against TSHR were detected by a commercially available 3rd generation enzyme immunoassay provided by RSR Limited, as described above for human serum samples. The assay was performed using 30 µL 1:10 (PBS) diluted serum in at least double determination.

Calculations and statistical analysis

All curves were fitted by SigmaPlot by using a 4-parameter logistic curve, which served to calculate EC_{50} and

IC_{50} values. Mean EC_{50}- and IC_{50}-values from several curves and corresponding CIs were calculated on the basis of decadic logarithms of concentration values. Results of *in vivo* experiments were compared by ANOVA when appropriate.

Results

Characterization of the temperature-sensitive and -insensitive forms of TSHR-His

Expression and purification

To provide the temperature-sensitive TSHR protein as ligand for human anti-TSHR antibodies and the temperature-insensitive form, CHO cells were stably transfected with the transgene expressing the his-tagged extracellular subunit A (1–289 aa) of the human TSHR. Appropriate cell culture conditions at 27°C or 37°C, respectively, allowed for a relatively more abundant production of either temperature-sensitive or -insensitive TSHR-His proteins. For TSHR-Fc, the production was adapted to flask culture and production at 27°C to dominantly produce the temperature-sensitive form (please see Methods).

Characterization of the binding to M22

In order to characterize the binding of the preparations of TSHR-His to M22, the human anti-TSHR antibody recognizing the active (here temperature-sensitive) form of the extracellular domain, we incubated various concentrations of temperature-sensitive TSHR-His and of conventionally prepared, temperature-insensitive TSHR-His or of TSHR-Fc with the immobilized M22 antibody and detected binding with specific POD antibody conjugates. The binding curves are shown in Fig. 1, which illustrates EC_{50} values of 0.24 µg/mL (corresponding to 5.7 nmol/L) for temperature-sensitive TSHR-His, and of 1.75 µg/mL (corresponding to 42 nmol/L) for temperature-insensitive TSHR-His, and of 0.96 µg/mL (corresponding to 8.6 nmol/L) for the temperature-sensitive form of TSHR-Fc used in this study. We also studied a small batch of TSHR-Fc which was incubated at 37°C for 24 h to induce a shift to the predominantly temperature-insensitive form. This affinity of this 'stressed' batch to M22 was 48 µg/mL (430 mmol/L), and hence, 50-fold lower than the temperature-sensitive form. This batch was studied further by ultracentrifugation.

The comparability of using monoclonal anti-His antibodies to detect TSHR-His versus polyclonal
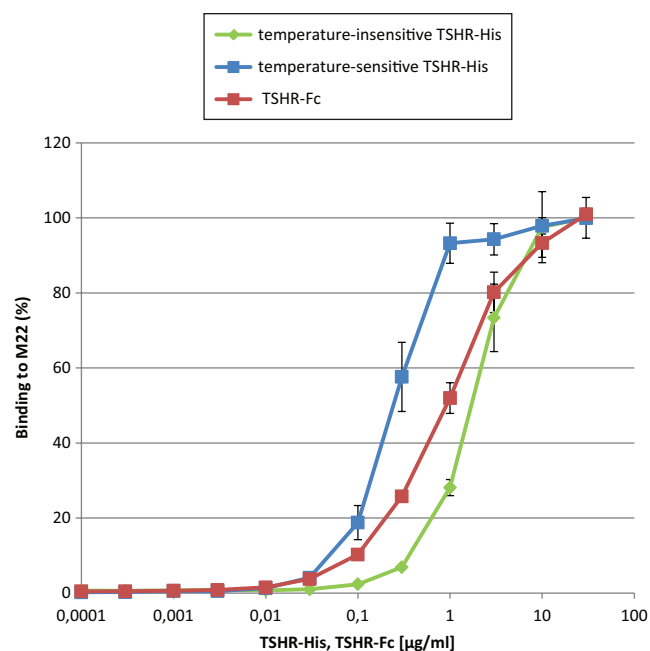

Figure 1

Binding of temperature-sensitive or temperature-insensitive TSHR-His or TSHR-Fc to immobilized anti-TSHR antibody M22. Response curves show the binding capacity of increasing concentrations of temperature-sensitive TSHR-His or TSHR-Fc in comparison to temperature-insensitive TSHR fusion proteins. Means of three independent experiments with S.D. are plotted. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.

anti-TSHR antibodies from TSHR-immunised mice was ascertained by using both in a head-to-head comparison assay for TSHR-His. A direct detection with anti-human Fc antibodies was not possible because of confounding signals elicited by the Fc part of the M22 antibody. The EC_{50} value of 0.24 µg/mL (corresponding to 5.7 nmol/L) for temperature-sensitive TSHR-His determined with the anti-His antibody was comparable to the EC_{50} determined with the murine polyclonal anti-TSHR antibodies (0.33 µg/mL, corresponding to 7.13 nmol/L).

Functional characterization: Effects of TSHR preparations on human anti-TSHR antibody-induced intracellular cAMP formation

To determine the functionality of temperature-sensitive and -insensitive TSHR-His and TSHR-Fc, we investigated their ability to inhibit M22-triggered human TSHR-mediated intracellular cAMP formation in JP2626 CHO cells, expressing the human TSHR. Figure 2 shows that temperature-sensitive TSHR-His inhibits M22-dependent cAMP formation with an IC_{50} value of 0.84 µg/mL (corresponding to 20 nmol/L) in this assay. Higher concentrations of the temperature-insensitive form of TSHR-His were required to obtain the same
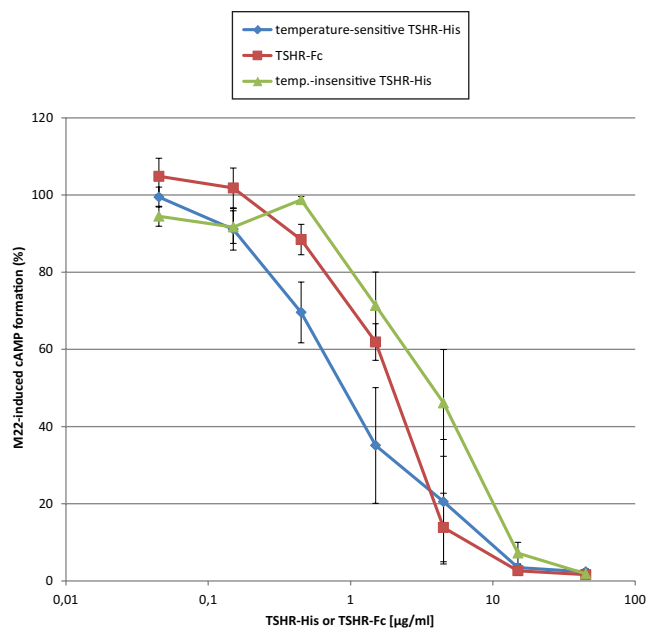
Figure 2

Effects of temperature-sensitive or temperature-insensitive TSHR-His or TSHR-Fc on cAMP formation induced by the monoclonal anti-TSHR antibody M22 in TSHR-expressing test cells. The concentration-dependent effects of temperature-sensitive and -insensitive TSHR-His or TSHR-Fc on intracellular cAMP formation were analyzed in JP2626 CHO cells expressing the human TSHR. TSHR was stimulated with monoclonal anti-TSHR antibody M22 (100 ng/mL final concentration). Concentration-dependent inhibition of the anti-TSHR antibody triggered stimulation of cAMP formation was most effective using temperature-sensitive TSHR-His. Results are shown as means of five experiments with S.E.M. A full colour version of this figure is available at https://doi.org/10.1530/JOE-20-0061.

inhibitory effect (IC_{50} value of 4.0 µg/mL, corresponding to 96 nmol/L). TSHR-Fc resulted in an IC_{50} value of 1.77 µg/mL, corresponding to 16 nmol/L.

Functional characterization in Graves' disease patients' blood samples

In addition to analyzing the effect on cAMP formation induced by the well-characterized, high-affinity monoclonal anti TSHR antibody M22, we characterized the capacity of the temperature-sensitive TSHR-His and TSHR-Fc to block cAMP formation induced by polyclonal antibodies in the serum of GD patients.

The presence of functionally active anti-TSHR antibodies in the GD patient sera used above was verified by investigating their stimulatory activity as the capacity of human serum samples to induce an increase in TSHR-dependent cAMP levels using JP2626 CHO cells. Many of the third generation-identified Graves' patient samples

also led to a significant increase in TSHR-dependent cAMP formation above baseline , determined to be 6 pmol cAMP/mLL.

Reassessment of the 48 suspected GD patients by third-generation assay showed that four did not fulfil GD and were therefore used as hyperthyroid, non-GD controls in this study. Of the remaining 44 GD samples, a significant TSHR-dependent cAMP increase above baseline occurred with 32 serum samples in TSHR-expressing test cells. Of these, 30 were inhibited by 25% or more by maximum concentrations of 100 µg/mL of both, TSHR-His or TSHR-Fc, respectively, corresponding to a response rate of 93% of both proteins in relation to those samples which elicited TSHR-dependent cAMP formation. Despite the overall broad GD population, an *ex vivo* effect of TSHR-His or TSHR-Fc was detectable in 70% of samples (data not shown).

A full characterization of concentration-response curves including EC_{50} values was done in 10–11 patient samples, which were randomly selected from the available GD serum samples. Figure 3A and B show that both temperature-sensitive TSHR-His and TSHR-Fc are capable to efficiently and dose-dependently reduce the cAMP formation induced by any of 11 Graves' patient sera. For TSHR-His, the IC_{50}-values varied between 0.57 and 13 µg/mL, corresponding to a range between 13.7 and 312 nmol/L. For TSHR-Fc, the IC_{50} values varied between 0.93 and 92 µg/mL, corresponding to a range between 8.37 and 828 nmol/L.

Figure 4 shows the mean inhibitory effects of temperature-sensitive TSHR-His or TSHR-Fc on TSHR-dependent cAMP formation induced by the 11 and 10 different GD patient serum samples. The resulting IC_{50} value was determined to be at 2.84 µg/mL (corresponding to 68 nmol/L) for TSHR-His and 3.29 µg/mL (corresponding to 29.6 nmol/L) for TSHR-Fc by graphical analysis of the mean value curve using SigmaPlot. This value corresponded fairly well to the mean value calculated from individually fitted IC_{50}-values of the single patient curves: for TSHR-Fc: 87 nmol/L, with a 95% CI of 19–400 nmol/L; for TSHR-His: 5.6 µg/mL, corresponding to 50.6 nmol/L, with a 95% CI of 1.6–1600 nmol/L.

Diagnostic ELISA for the identification of GD patients

In the previous section, we have shown that temperature-sensitive TSHR His bound/inhibited M22 with low EC_{50}-/IC_{50}–values and targeted functional anti-TSHR antibodies. Therefore we considered this protein as a
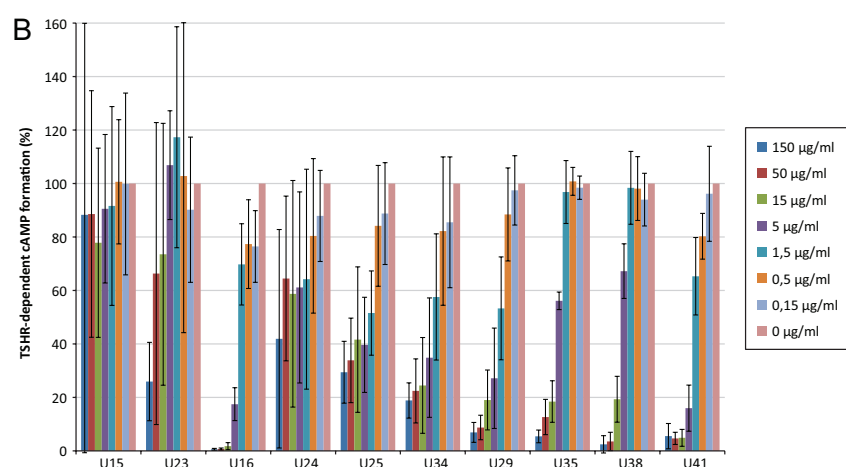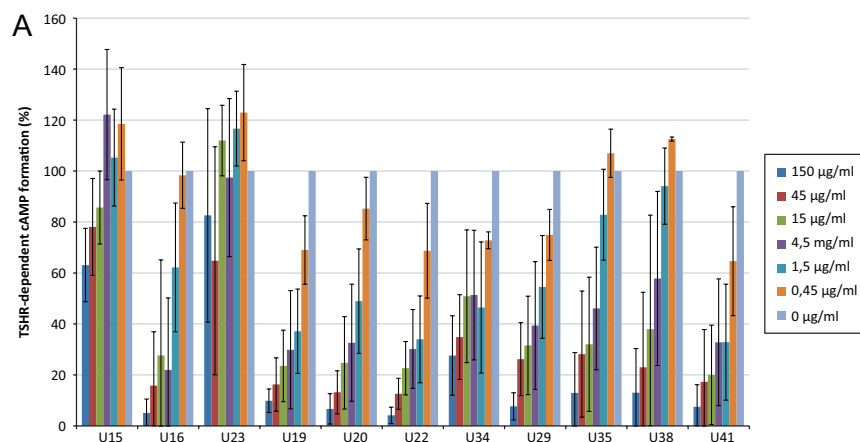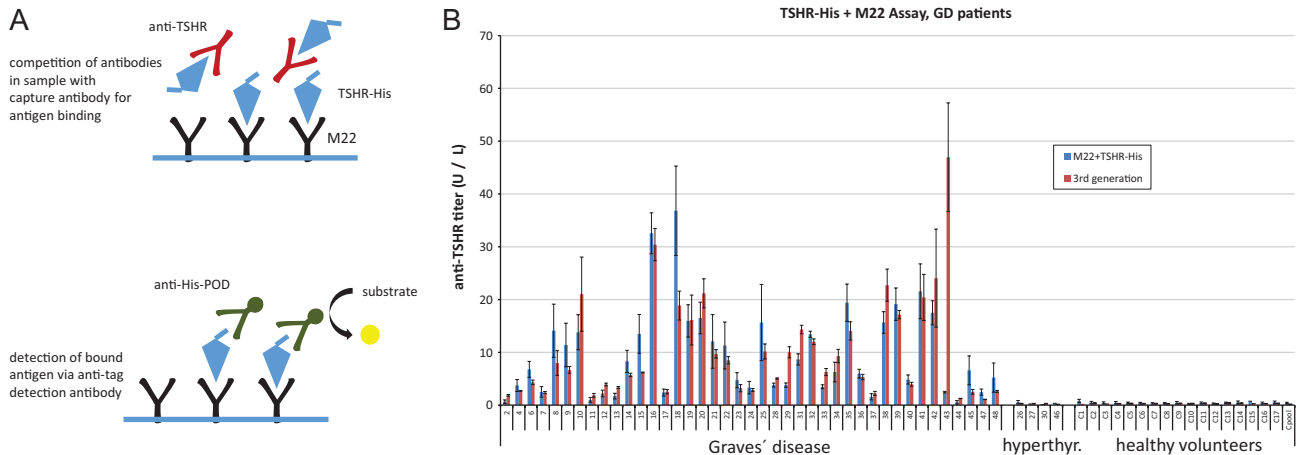
Figure 3

Effects of temperature-sensitive TSHR-His (A) or TSHR-Fc (B) on cAMP formation induced by Graves' disease patient serum samples containing anti-TSHR antibodies. The concentration-dependent effect of temperature-sensitive TSHR-His or TSHR-Fc on the intracellular cAMP formation was analysed in JP2626 CHO cells expressing the human TSHR. TSHR was stimulated with eleven serum samples from distinct GD patients (diluted 1:5). Codes on x axis indicate individual patients. Results are shown as means of triplicate analyses with s.e.m. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.
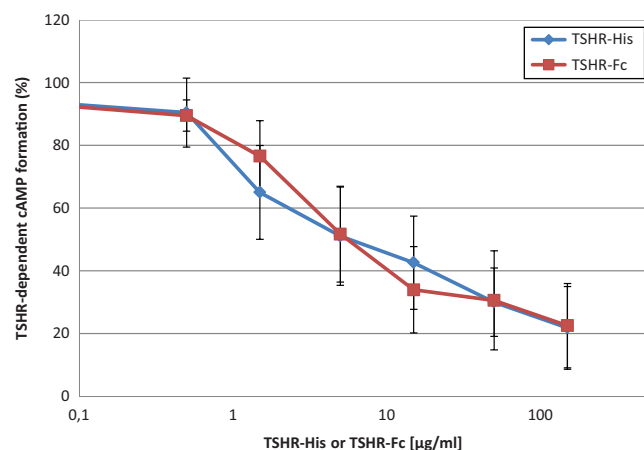
Figure 4

Mean inhibition of anti-TSHR antibody-induced intracellular cAMP expression by TSHR preparations in GD patient samples. Mean values ± s.e.m. of all 11 investigated GD patient samples: inhibition of anti-TSHR antibody-induced intracellular cAMP levels by temperature-sensitive TSHR-His (blue symbols) or TSHR-Fc (red symbols). A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.

suitable tool to be used as a soluble probe in a competition ELISA, designed to measure anti-TSHR antibody titer from sera of GD patients. In comparison to the third generation assay, this new 'M22-TSHR-His' ELISA format inverted the position of the binding partners (see Fig. 5A for illustration). The human anti-TSHR antibodies from GD patients inhibited the binding of the immobilized human monoclonal thyroid stimulating antibody M22 to the soluble temperature-sensitive TSHR-His protein.

Figure 5B shows the direct comparison of the anti-TSHR titres of 41 serum samples from GD patients, 4 control serum samples from (latently) hyperthyroid patients without GD and 17 healthy controls, which were obtained in the third generation assay (with lower sample volumes) and in the new assay format, respectively. In order to ensure comparability, the identical standard samples provided by the third generation assay kit were used in both assays. Sharing the third generation assay cut-off value of 0.4 U/L, 96.8% serum samples were classified consistently – in detail, 39 samples with GD were recognized as positive and all

Figure 5

Comparison of titer values of GD patients measured by third generation assay vs M22-TSHR-His assay. (A) Schematic overview of the novel 'M22 + TSHR-His' ELISA method. (B) The competitive activity of anti-TSHR antibodies was analysed in 41 serum samples of GD patients ('Graves' disease') and 17 control samples of healthy volunteers (C1–C17). 'C Pool' indicates an additional measurement of all C samples together. In addition, four samples of hyperthyroid patients in whom no evidence of Graves' disease was detected served as another control group ('hyperthyroidism'). Each bar represents the mean ± standard deviation (SD) of duplicate determinations for either the novel 'M22 + TSHR-His' ELISA method (blue bars) or the current gold standard assay ('3rd generation'; red bars). A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.

17 control samples of healthy volunteers were recognized as negative. Similarly, four hyperthyroid control patients in whom no evidence of GD was detected were also correctly recognized as negative. The titer values of both assays showed a high consistency. Comparing the values measured in the samples of the same subjects by using the third generation assay and the new assay, the correlation coefficient amounted to $R = 0.57$. This ELISA might thus be alternatively used for the determination of TSHR autoantibodies in human serum.

Analysis of TSHR-Fc by ultracentrifugation

The presence of multimers and monomers of 'stressed' (37°C for 24 h) vs unstressed dimeric TSHR-Fc was investigated after ultracentrifugation. The percentage of dimers was very similar after stressed and non-stressed conditions, and after stressing some monomers change to forms with higher molecular weights (data not shown).

Mouse studies *in vivo*

Ad-TSHR-immunized mice were randomly assigned to receive epicutaneous patches containing either 0.3 or 0.6 mg/kg body weight TSHR-Fc or vehicle control. This therapy was started 1 week after the fourth immunization by epicutaneous patches, and then continued at 4-weekly intervals. No animals were prematurely taken out of the study after randomization, and the prespecified

protocol was completed in all animals. Results reflect the respective means of all values of all randomized animals in the groups. Tolerability of patches had been tested in a previous pilot study.

Thyroid sizes, as determined from serial sections

Thyroid volumes (mm³) were determined from the sum of the areas of each section over the whole cutting region (between slides 5 and 10, depending on respective size of the thyroid gland) multiplied by the slice thickness of 0.5 mm. This macroscopic investigation showed clearly increased thyroid sizes in mice which had received nine immunizations of Ad-TSHR289 (Fig. 6), compared to the healthy and GFP-immunized control groups. There was no statistically significant difference of thyroid sizes between Ad-TSHR-immunized mice exposed to epicutaneous TSHR-Fc and untreated controls.

Determination of thyroxin serum levels

Thyroxin (T4) levels did not differ between groups at study start, and mean T4 levels in eligible animals of the Ad-TSHR289-immunized groups were significantly higher than controls at week 11 (start of therapy, Fig. 7), and exceeded the normal T4 value range, which was defined as 5,97–6.81 μg/dL in our laboratory (means ± 2 s.e. of control mice). There was no statistically significant difference of T4 values between Ad-TSHR-immunized mice exposed to epicutaneous TSHR-Fc and untreated controls.
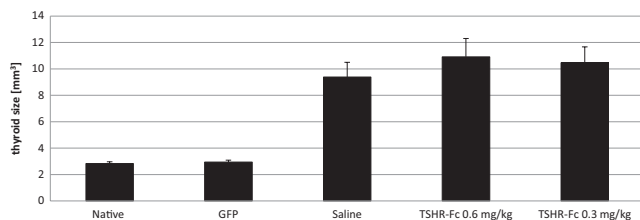
Figure 6

Effects on macroscopically measured thyroid size. The effects of the TSHR-Fc fusion protein on thyroid sizes were investigated at the end of the experiment. The measurements were carried out in Ad-TSHR-immunized mice treated by either 4-weekly epicutaneous patch administrations of vehicle (0.9% NaCl, 'saline', *n* = 12 mice), or administrations of 0.3 mg/kg (12 mice) or 0.6 mg/kg body weight (11 mice) of TSHR-Fc. In addition, Ad-GFP-immunized control mice ('GFP', *n* = 5 mice) and age-matched immunologically naïve unimmunized mice ('native', 5 animals) were investigated. The mean thyroid sizes (mm³) are shown with s.e.m. There were no significant differences between Ad-TSHR-immunized groups as determined by AVOVA testing.

ECG to determine heart rates

Starting from the third immunization, a significant increase in heart rate in the hyperthyroid Ad-TSHR289 immunized group was observed (Fig. 8). In contrast, heart rate in the native, healthy group or in the GFP immunized control group was only mildly increased at older age, but did not change significantly.

Upon consecutive Ad-TSHR289-immunizations, a further strong increase in heart rate in the hyperthyroid vehicle-treated group was observed. There was no statistically significant difference of heart rate between Ad-TSHR-immunized mice exposed to epicutaneous TSHR-Fc and untreated controls.

Anti-TSHR antibody titers

Anti-TSHR antibodies were determined from serum samples by investigating the ability of the respective mouse sera to inhibit the binding of the monoclonal Graves' patient antibody M22 to the TSHR ('3rd generation ELISA). High titers were detected in all Ad-TSHR289-immunized animals (Fig. 9). There was no statistically significant difference of anti-TSHR antibody titers between Ad-TSHR-immunized mice exposed to epicutaneous TSHR-Fc and untreated controls, but the TSHR-Fc-treated groups trended to develop even higher titers than the control group.

Discussion

This manuscript characterizes the EC_{50}- and IC_{50}-values of temperature-sensitive forms of TSHR A domain fusion proteins – His-tagged TSHR and TSHR-Fc – based on measurements of concentration-dependent binding and functional effects on anti-TSHR antibodies. We show that IC_{50} values to inhibit monoclonal M22-dependent cAMP formation of either His-tagged TSHR (which contains only one TSHR A domain) or an TSHR-Fc fusion protein (which contains two TSHR A domains at relatively fixed steric orientation) are observed at lower nanomolar concentrations. In addition, both proteins inhibit cAMP formation induced by various Graves' patient sera at similar concentrations. However, epicutaneous administration of one of these fusion proteins, TSHR-Fc, did not improve Graves' disease parameters in a long-term mouse model.

Chazenbalk *et al.* (2001) had shown effects of a similar TSHR construct at a maximum concentration which was comparable to our results. The high efficiencies of both, the temperature-sensitive His-tagged TSHR and TSHR-Fc in blocking the anti-TSHR antibody-induced stimulation of the TSHR might not only qualify the proteins as soluble probes, but also as potential anti-TSHR antibody scavengers or immune modulating agents *in vivo*.

Both proteins react with autoantibodies of blood samples of a large majority of GD patients. Out of a study
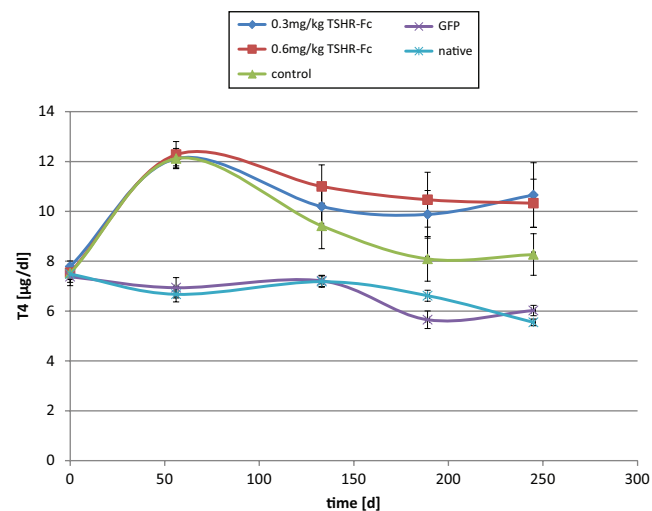
Figure 7

Effect on serum thyroxin (T4) levels. The effects the TSHR-Fc fusion protein on serum thyroxin levels were evaluated. The measurements were carried out in serum samples of Ad-TSHR-immunized mice treated by either 4-weekly epicutaneous patch administrations of vehicle (0.9% NaCl, 'saline, *n* = 12 mice), or administrations of 0.3 mg/kg (12 mice) or 0.6 mg/kg body weight (11 mice) of TSHR-Fc. In addition, Ad-GFP-immunized control mice ('GFP', *n* = 5 mice) and age-matched immunologically naïve unimmunized mice ('native', 5 animals) were investigated. Data are represented as means ± s.e.m. There were no significant differences between Ad-TSHR-immunized groups compared to the untreated control as determined by AVOVA testing. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.
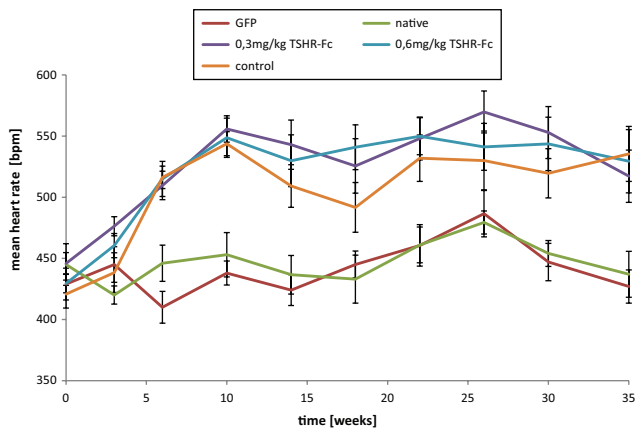
Figure 8

Effect on heart rates. The effects the TSHR-Fc fusion protein on heart rates at various times were evaluated in all animals. The measurements were carried out in Ad-TSHR-immunized mice treated by either 4-weekly epicutaneous patch administrations of vehicle (0.9% NaCl, 'saline, n = 12 mice), or administrations of 0.3 mg/kg (12 mice) or 0.6 mg/kg body weight (11 mice) of TSHR-Fc. In addition, Ad-GFP-immunized control mice ('GFP' , n = 5 mice) and age-matched immunologically naïve unimmunized mice ('native, 5 animals) were investigated. Data are represented as mean ± S.E.M. There were no significant differences between Ad-TSHR-immunized groups as determined by AVOVA testing. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.

population of 44 reconfirmed GD patients, 32 serum samples elicited measureable cAMP formation – almost all of these showed significantly decreased TSHR-dependent cAMP formation upon exposure to either 100 μg/mL TSHR-His or TSHR-Fc.

Generally, the described assays and compounds are based on previous reports on preparations of the TSHR A-subunit, which can be purified from overexpressing cell culture supernatants in two different forms. The temperature-sensitive ('active') form neutralized the TSH binding inhibitory activity of Graves' sera, whereas the temperature-insensitive ('inactive') form was not reactive with sera from Graves' patients (Chazenbalk *et al.* 1999, 2001, Schwarz-Lauer *et al.* 2002). Based on their crystal structures, M22 (Sanders *et al.* 2007) and 3BD10 (Chen *et al.* 2015) should be able to bind simultaneously to the monomeric form of the TSHR A-subunit. Chen *et al.* (2015) thus concluded that the monomeric A-subunit cannot explain the reciprocally exclusive anti-TSHR antibody/ M22 and 3BD10 binding, but instead hypothesized that TSHR-stimulating antibodies and 3BD10 bind to A-subunit multimers with different quaternary structure/valency. In silico analyses of the group suggested that stimulatory anti-TSHR antibodies bind to trimers, whereas 3BD10 binds to dimers (Chen *et al.* 2015).

Rapoport *et al.* (2015) picked up this concept and systematically (re)investigated the binding characteristics

of sera from Graves' patients and from mice that developed hyperthyroidism due to repeated immunizations with TSHR A-subunit-expressing adenovirus. The group confirmed earlier results that pathologic sera from Graves' patients inhibited binding of TSH to temperature-sensitive TSHR, but only rarely bound to the temperature-insensitive TSHR A-subunit. They further showed that sera of hyperthyroid mice recognized both forms of the TSHR A-subunit, and concluded that pathogenic autoantibody affinity maturation in Graves' disease is probably driven by A-subunit multimers, not monomers.

Our analysis of the (dimeric) temperature-sensitive TSHR-Fc fusion protein showed that this dimeric protein occurs mainly as such in solution, but partly assembles spontaneously into dimers of these dimers. Stressing conditions do not have an impact on this condition, but impact on binding to the antibody M22, so that at least with the very active and high-affinity binding TSHR-Fc, the hypothesis of Rapoport *et al.* (2015) about the importance of trimers versus dimers does not seem to be relevant. Our approach, however, is limited by the following considerations: The fixed orientation of two A-subunits attached to an Fc is not exactly like the random association
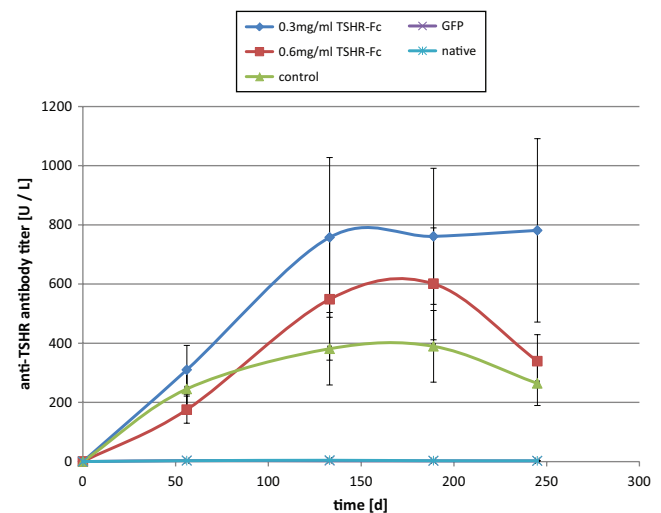

Figure 9

Effect on anti-TSHR antibody titers. The effect of the TSHR-Fc fusion protein on time course of anti-TSHR titers, as measured by third generation ELISA, in which serum samples are used to determine inhibition of M22-binding to coated plates. The measurements were carried out in Ad-TSHR-immunized mice treated by either 4-weekly epicutaneous patch administrations of vehicle (0.9% NaCl, 'saline', n = 12 mice), or administrations of 0.3 mg/kg (12 mice) or 0.6 mg/kg body weight (11 mice) of TSHR-Fc. In addition, Ad-GFP-immunized control mice ('GFP', n = 5 mice) and age-matched immunologically naïve unimmunized mice ('native, 5 animals) were investigated. Data are represented as mean ± S.E.M. There were no significant differences between Ad-TSHR-immunized groups as determined by AVOVA testing. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0061.

of free, unattached A-subunits. Also, temperature-sensitive TSHR fusions may represent a mixture of active and inactive forms: their amounts required to fully neutralize anti-TSHR-antibodies (Fig. 3) were higher than those required by purified active A-subunits (Chazenbalk *et al.* 2001) – some of the preparations may therefore be inactive. The temperature-insensitive TSHR-His preparation contained conformations that are still recognized by anti-TSHR antibodies specific for the 'immunologically active' conformation (M22 and autoantibodies of the GD sera) (Figs 1 and 2), whereas purified inactive A-subunits were not recognized by these antibodies (Chazenbalk *et al.* 2001).

Our results show that a high affinity interaction of TSHR A subunit with the anti-TSHR antibody M22 occurs, regardless if it is C-terminally extended with a His tag or dimerized via Fc.

In comparison to the gold standard third generation assay (Smith *et al.* 2004), we established a new ELISA format which inverts the position of the binding partners. The human anti-TSHR antibodies from GD patients inhibit the binding of the immobilized human monoclonal thyroid stimulating antibody M22 to the soluble temperature-sensitive TSHR-His protein. This assay configuration avoids the highly challenging purification procedure of the membrane-bound TSHR protein. A second beneficial effect is that the minimal serum volume is reduced to 8% of that used in the third generation assay.

It had been known before that addition of nanogram quantities of the temperature-sensitive TSHR A domain to Graves sera can fully neutralize the ^{125}I-TSH binding inhibition (Chazenbalk *et al.* 1999, Rapoport *et al.* 2015), anti-TSHR antibodies (Chazenbalk *et al.* 2002) and M22-induced intracellular cAMP formation (Mizutori *et al.* 2009).

Here, we show and reconfirm that the temperature-sensitive form of His-tagged TSHR inhibits M22 binding to TSHR and human Graves' serum-induced TSHR-dependent cAMP induction in test cells at nanomolar IC_{50} values. In addition, we show that this is also true for temperature-sensitive form of a TSHR-Fc fusion protein which expresses two TSHR A domains at a fixed steric conformation.

However, epicutaneous administration of TSHR-Fc did not result in any beneficial effect on a long-term disease model *in vivo*. The results of this study partially corroborate those after repeated i.v. administration of a fusion protein of the TSHR A domain with Fc in immunologically naïve mice: 2 animals developed anti-TSHR titers after treatment with 1 mg/kg TSHR-Fc, and

suffered from signs of allergy (Holthoff *et al.* 2017). These findings are in line with those after single administration of TSHR A domain (Misharin *et al.* 2009) or 4-fold administration of a nanoparticle-coated TSHR A domain (McLachlan *et al.* 2019) in Graves' mouse models, but are in contrast to those obtained after administration of TSHR-derived cyclic peptides, which did not induce any immune response and can re-establish tolerance toward the antigen in immunized mice (Holthoff *et al.* 2017, Faßbender *et al.* 2019), improving symptoms of GD within 3–4 months after starting these therapies.

As another antigen-specific therapy to induce tolerance to the TSHR in patients with GD, T cell epitope-derived linear peptides have also been identified using immunized HLA-DR3 transgenic mice (Jansson *et al.* 2016, 2018). A combination of such peptides (Jansson *et al.* 2018) has yielded promising results in a first clinical trial in patients with GD (Pearce *et al.* 2019).

In summary, despite high affinity to bind to Graves' patients anti-TSHR auto-antibodies, epicutaneous administration of TSHR-Fc did not exert a beneficial effect on hyperthyroidism, tachycardia and other *in vivo* read-outs in a long-term mouse model of Graves' disease induced by immunization with a recombinant adenovirus encoding TSHR A in mice *in vivo*. These results should therefore guide further clinical development of antigen-specific therapies of the disease. They rather discourage to use the full Graves' auto-antigen to achieve this goal.

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