

## Prolonged TSH Receptor A Subunit Immunization of Female Mice Leads to a Long-Term Model of Graves' Disease, Tachycardia, and Cardiac Hypertrophy

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A transient model for human Graves' disease was successfully established in mice using up to 3 immunizations with recombinant adenovirus expressing the extracellular A-subunit of the human TSH receptor (TSHR) (Ad-TSHR). We studied extension of adenovirally induced TSHR A-subunit immunization in mice by using a novel protocol of long-term 3- and 4-weekly injections. Generation of TSHR binding stimulatory antibodies (capacity to stimulate cAMP activity in TSHR-expressing test cells), goiter, and histological thyroid alterations were maintained for at least 9 months in all Ad-TSHR-immunized mice. In response to injection of  $10^{10}$  plaque-forming units of Ad-TSHR, also elevated mean serum  $T_4$  levels were observed throughout the study. Moreover, cardiac organ involvement (tachycardia and hypertrophy) were consistently observed in these mice. Higher doses of Ad-TSHR ( $10^{11}$  plaque-forming units) did not produce consistent elevation of  $T_4$  and were not associated with a clear increase in heart rate vs controls, probably because these high doses provoked an immune response-induced tachycardia on their own. In summary, a long-term model of Graves' disease induced by a relatively simple protocol of continuing monthly immunizations should allow to investigate long-term disease mechanisms and may possibly obviate the need for more complicated disease models. Moreover, the clinical outcome predictor of tachycardia and cardiac involvement was reliably detected in the model. (*Endocrinology* 156: 1577–1589, 2015)

**G**raves' disease is an antibody-mediated autoimmune condition targeting the TSH receptor (TSHR) in the thyroid gland, resulting in hyperthyroidism (1). A clinical feature of all forms of hyperthyroidism is cardiac involvement, which is directly caused by the action of thyroid hormones on nuclear receptors within the myocardium (2). Hence, contractility is increased, and a hypertrophic form of cardiomyopathy (2), as well as tachycardia is observed in these patients, and the increase in heart rate is a reliable marker of disease severity (2, 3). Atrial fibrillation (AF) occurs in 5%–15% of patients (2), and palpitations are among the most reported symptoms defining disease burden. Increased cardiovascular morbidity and mortality

have been reported in patients with both overt or subclinical hyperthyroidism (4–8). Hyperthyroid patients suffering from Graves' disease are at especially increased cardiovascular risk (reviewed in Ref. 8).

Mouse models of Graves' disease have raised considerable interest. They allow to better understand the disease entity and to investigate novel therapeutic approaches. A disease model for human Graves' disease was successfully established with up to 3 immunizations with recombinant adenovirus expressing the full-length human TSHR (9) and soon reconfirmed in further studies using the extracellular A-subunit of the TSHR (10, 11). Subsequent studies concerned variations of adenoviral titers which were

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Abbreviations: Ad-GFP, adenovirus containing only the reporter gene GFP; Ad-TSHR, adenovirus expressing the extracellular A-subunit of the human TSHR; AF, atrial fibrillation; CHO, Chinese hamster ovary; ECG, electrocardiogram; GFP, green fluorescent protein; LSD, least significant difference; pfu, plaque-forming units; RM-ANOVA, ANOVA for repeated measurements; TSHR, TSH receptor.

used for the immunizations (12) or injection of dendritic cells (reviewed in Refs. 13, 14). The use of adenovirus was more effective than use of plasmid transfection in most studies, with the exception of a novel protocol based on muscular electroporation using hindlimb electrode needle pairs (15–17), which allowed to detect stimulating anti-TSHR antibodies up to 32 weeks after 4 such electroporations. This protocol also holds promise to investigate orbitopathy and retrobulbar inflammation and fibrosis in these mice.

Prolongation of the protocol based on 3 adenovirally induced immunizations over 6 weeks, and measurements after 20 weeks instead of 10 weeks also led to disease induction (18). The immunizations with recombinant TSHR were studied in both, wild-type or TSHR transgenic mice. Although anti-TSHR binding inhibition was consistently observed after 20 weeks in these virus-immunized mice, the potency of the resulting antibodies to stimulate cAMP considerably diminished or even disappeared during the course of the experiment (50% positive at the end of the experiment), and no T<sub>4</sub> elevation nor thyrocyte hyperplasia was observed (18).

In this study, we have extended the existing model of adenovirally induced TSHR immunization by using a novel protocol, in which regular injections are continued for 9 months, in order to permanently boost antibody production in mice. This protocol was established in parallel to a previous study, which successfully established a long-term disease model of cardiomyopathy caused by anti-β<sub>1</sub> receptor antibodies in rats (19, 20). In the current study, we have combined the approach used by pivotal previous publications (10, 11), which used 3 3-weekly immunizations, and have extended this initial phase by a maintenance phase with further regular 4-weekly boosts. Because of previous reports on various adenoviral titers, we decided to include 2 groups of 10<sup>10</sup> and 10<sup>11</sup> plaque-forming units (pfu) of recombinant adenovirus expressing either TSHR or control green fluorescent protein (GFP).

We show that this protocol does not only lead to generation of TSH binding antibodies but also that these stimulate cAMP activity and induce thyroid hyperplasia and marked histological alterations for at least 9 months. In addition, we show that immunization with 10<sup>10</sup> pfu led to consistent T<sub>4</sub> level increases. We also show that in this long-term model of hyperthyroidism, tachycardia and cardiac hypertrophy were consistently observed after immunization with 10<sup>10</sup> pfu and, thus, show that the model allows to measure these parameters, which especially impact on prognosis of affected patients.

## Materials and Methods

### Recombinant adenovirus

The DNA sequence coding for the first 289 amino acids of the human TSHR (21, 22) were synthesized by Eurofins. Then, the sequence was cloned into the Microbix Admax adenovirus expression system plasmid pDC516 using *EcoRI* and *SalI* restriction sites. This shuttle plasmid pDC516 was cotransfected with pBHGfrt(del)E1,3FLP (genome plasmid) into HEK293A cells (Invitrogen). Integrity of the plasmids was tested by sequencing.

HEK293A cells were seeded in 10 cm cell culture dishes one day before cotransfection with the 2 plasmids. CaCl<sub>2</sub> was used for transfection. The culture medium (DMEM + 10% fetal bovine serum + phosphate saline) was exchanged 24 hours after transfection, and the cells were incubated at 37°C/5% CO<sub>2</sub> until first viral plaques became visible. This system results in recombinant replication-deficient E1 and E2-deficient adenovirus type 5. A control adenovirus containing only the reporter gene GFP (Ad-GFP) was amplified and purified in the same manner.

After emergence of first viral plaques, respective HEK293A cells were grown, then completely detached from the flasks using a cell scraper. The resulting cell suspension was centrifuged at 1000g for 5 minutes. Purification of the adenoviruses was done using the Clontech Adeno-X Maxi purification kit according to manufacturer's protocol. To determine the viral particle titer of the purifications, samples were diluted in a ratio of 1:10 with PBS + 0.1% sodium dodecyl sulfate (SDS) and incubated for 10 minutes at 56°C. Subsequently, the OD at 260 nm was measured against PBS + 0.1% SDS (blank).

The resulting recombinant adenovirus was compared with a similar adenovirus expressing the extracellular A-subunit of the human TSHR (Ad-TSHR) virus expressing the A-subunit (1–269) of TSHR, which was kindly provided by Dr Rapoport, Cedars Sinai Research Institute, University of Los Angeles, CA. Both kinds of adenovirus were found to possess similar efficacy to produce anti-TSHR antibodies in mice after immunization using comparable virus titers.

### Animal studies

Female BALB/c mice were delivered from Charles River, at the age of 4–5 weeks, 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% relative humidity) in groups of 10 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 (nos. 55.2-1-54-2531-25-12), and carried out in accordance to the European Commission guidelines. All guidelines for care of animals were respected.

Mice were randomly assigned into verum immunization groups, receiving either 10<sup>10</sup> or 10<sup>11</sup> pfu of adenovirus carrying the A-subunit of the TSHR gene, of which 24 and 9 animals went through the complete protocol, or respective mock immunization groups, which received either 10<sup>10</sup> or 10<sup>11</sup> pfu GFP control virus (32 and 10 animals, respectively). In addition, age-matched immunologically naive mice (9 animals) were studied for comparison.

For immunization, mice were anesthetized 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  $\mu\text{L}$  each.

For blood withdrawal, mice were placed under infrared light for at least 30 minutes for dilatation of the tail veins, then moved to a restrainer. A total of 100  $\mu\text{L}$  of blood was withdrawn out of the left or right tail vein with a 27-G needle. Blood was centrifuged at 2400g for 15 minutes at room temperature, and serum was stored at  $-20^{\circ}\text{C}$ . 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 27-G needle and treated as mentioned above.

The protocol of the current study combined the approach used by pivotal previous publications (10, 11), which used 3 3-weekly immunizations (“initiation”), and extended this initial phase by a “maintenance” phase with further regular 4-weekly boosts until the ninth immunization. A detailed immunization schedule is shown in Figure 1A.

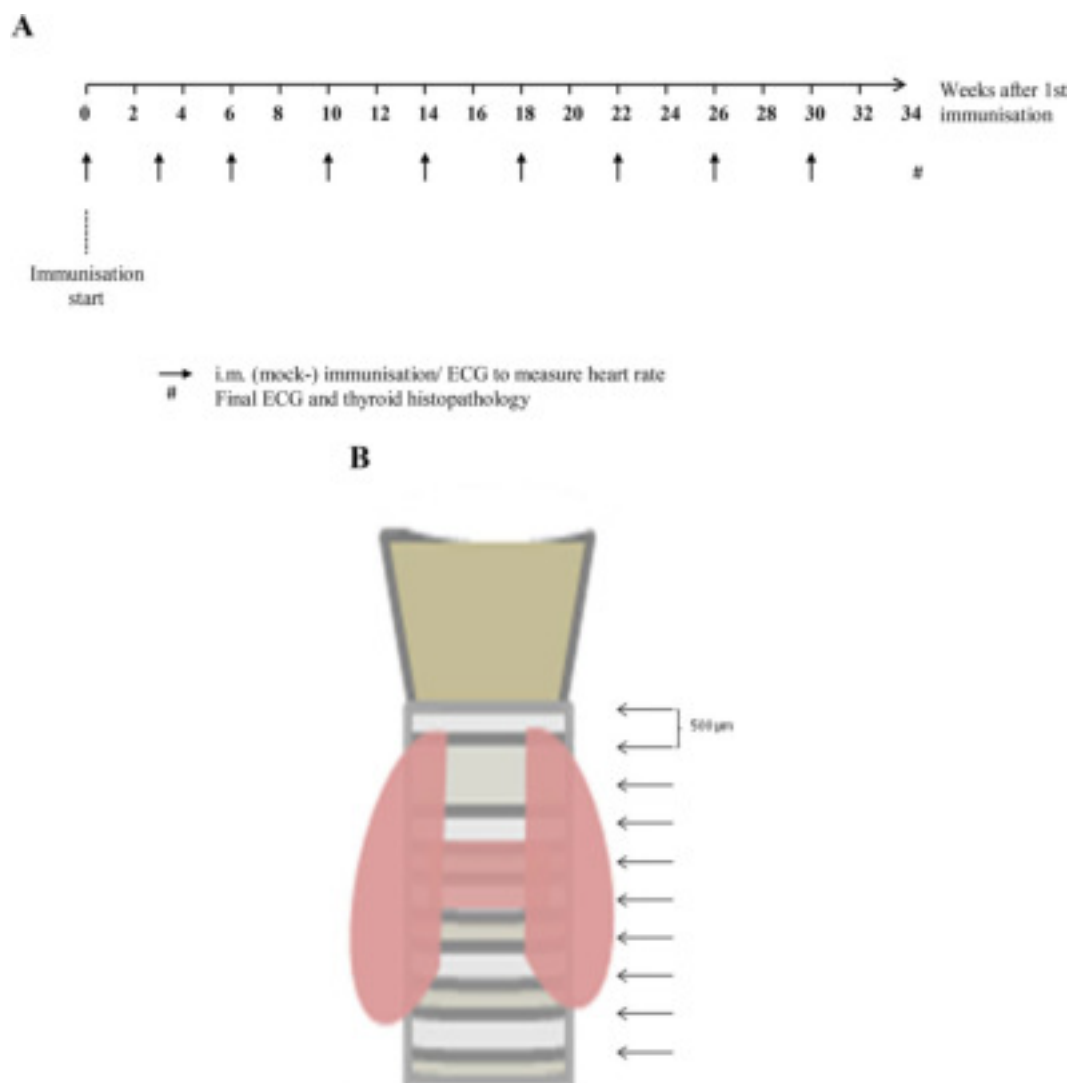
In addition to this main study, a previous pilot study compared 14 Ad-TSHR-immunized mice with 10 Ad-GFP-immunized mice and 6 native age-matched control mice over 20 weeks.

When mice were in anesthesia for immunization, their heart function was monitored with an electrocardiogram (ECG) (ECG amplifier module, Harvard Apparatus, Hugo Sachs Electronics) and recorded with a special software, which allows to determine the heart rate from the ECG reading (Hemodyn, Hugo Sachs Electronics). ECG was also performed in anesthesia before animals were euthanized for histological examination at the end of the study.

### Measurements in mouse sera

Anti-TSHR autoantibody titers and potency of antibodies to stimulate TSHR-dependent cAMP levels in test cells were determined before start of immunization (basal value), 56 days after first immunization, 133 days after first immunization, 189 days after first immunization, and at the end of experiment. Simultaneously, titers of anti-TSHR antibodies were determined. To this aim, 3 different assays were established:

- 1) Second generation assay. Inhibition of TSH binding was determined by a commercially available standard

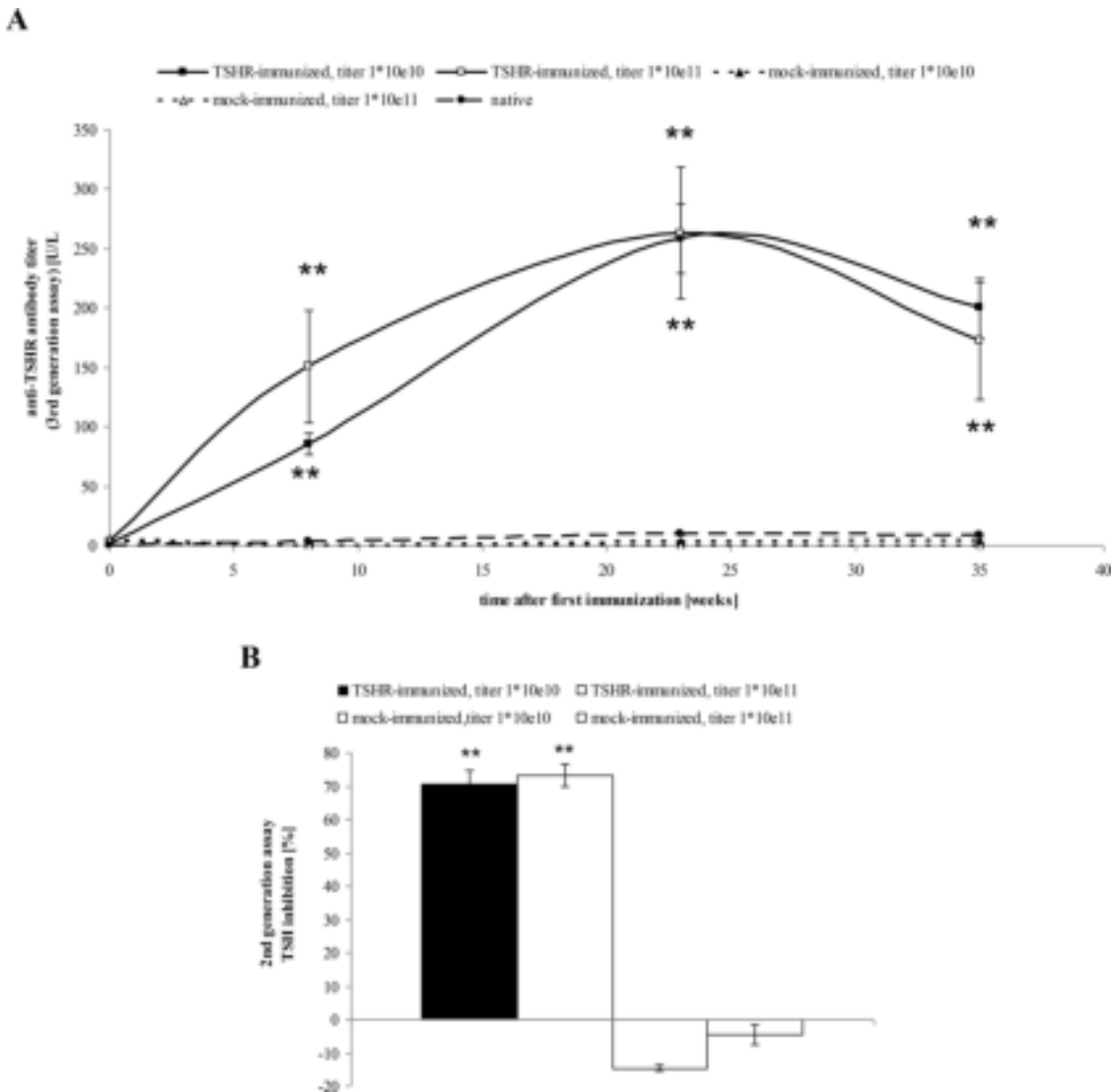


**Figure 1.** A, Time schedule of the study: immunizations. The panel shows the time course of immunizations and measurements. B, Cutting positions for thyroid sections. The arrows indicate the placement of sections at a distance of 500  $\mu\text{m}$ , which was oriented on the anatomical situs of surrounding organs.

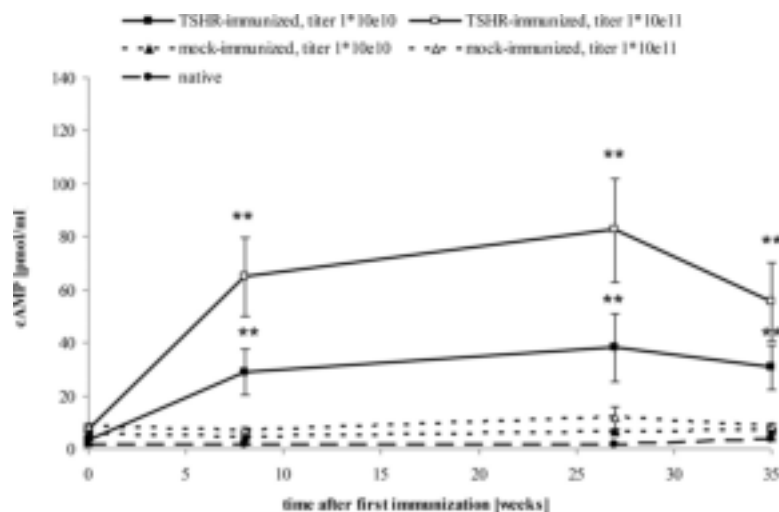
diagnostic assay used for human patients, as provided by RSR Ltd, according to the manufacturer's instructions.

2) Third generation assay. Antibodies against TSHR were also detected by a commercially available third generation enzyme immunoassay provided by RSR Ltd, in which the of the human Graves' patient M22-biotin monoclonal antibody and serum antibodies compete for binding sites on immobilized TSHR. The assay is also used in Roche's Cobas<sup>R</sup> assay (04388790) for electro-chemo luminescence immuno assay with minor modifications. The assay was performed using 30- $\mu$ L 1:10 (PBS) diluted serum in at least double determination according to the manufacturer's instructions.

3) Thyroid stimulating antibodies in the serum of hyperthyroid mice were analyzed by measuring cAMP generation in Chinese hamster ovary (CHO) cells JP2626 expressing the human TSHR (kindly provided by Dr Gilbert Vassart, Brussels, Belgium). CHO cells were seeded into 96-well plates (30000 cells per well) and incubated for 24 hours in DMEM (Invitrogen Ltd) containing 2% fetal calf serum. Then, DMEM was then removed and mice serum was diluted 1:8 in 40- $\mu$ L HBSS buffer (20mM HEPES, 1.26mM CaCl<sub>2</sub>, 5.33mM KCl, 0.44mM KH<sub>2</sub>PO<sub>4</sub>, 0.5mM MgCl<sub>2</sub>, 0.4mM MgSO<sub>4</sub>, 4.2mM NaHCO<sub>3</sub>, 5.6mM glucose, and 222mM sucrose; pH 7.2) supplemented with 1.5% BSA and 0.5mM isobutyl-1-methylxanthine (Sigma-Aldrich) and added to each well. After incubation for 2.5 hours at



**Figure 2.** Effect of immunization with adenovirus encoding the A-subunit of the TSHR on anti-TSHR antibody titers. The effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR on development of anti-TSHR antibodies was evaluated. A, Time course of anti-TSHR titers, as measured by third generation ELISA. The measurements were carried out in 24 animals receiving 10<sup>10</sup> pfu Ad-TSHR, 9 animals receiving 10<sup>11</sup> Ad-TSHR, 32 animals receiving 10<sup>10</sup> pfu Ad-GFP, and 10 animals receiving 10<sup>11</sup> pfu Ad-GFP. In addition, age-matched immunologically naive mice (9 animals) were investigated. B, Additionally, at the end of the study (time 35 wk), most animals were counterchecked with a second generation ELISA. For this analysis, only samples from 44 animals were available; blood volumes did not allow for measurements in the remaining animals. Data are represented as mean  $\pm$  SEM. Significance over time was tested by ANOVA of groups at given time points, and controlled by RM-ANOVA within 1 group, followed by LSD post hoc testing. \*\*, statistical significance ( $P < .005$ ) compared with the mock-immunized group.



**Figure 3.** Effect on cAMP stimulation in TSHR-expressing test cells, as determined in sera taken from the mice during the experiment. The effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR on the capacity of anti-TSHR antibodies to stimulate cAMP generation in CHO cells expressing the human TSHR was evaluated and compared with a mock-immunized group. Data are represented as mean  $\pm$  SEM. All measurements were carried out in 24 animals receiving  $10^{10}$  pfu Ad-TSHR, 9 animals receiving  $10^{11}$  Ad-TSHR, 32 animals receiving  $10^{10}$  pfu Ad-GFP, and 10 animals receiving  $10^{11}$  pfu Ad-GFP. In addition, age-matched immunologically naive mice (9 animals) were investigated. Significance over time was tested by ANOVA of groups at given time points and controlled by RM-ANOVA within 1 group, followed by LSD post hoc testing. \*\*,  $P < .005$  TSH-immunized group compared with the respective (titer-matched) mock-immunized group.

$37^{\circ}\text{C}$ , the cAMP release in the medium was measured in duplicates by a competitive immunoassay ELISA (EM-SCAMPL; Thermo Fisher Scientific).

Total  $T_4$  was measured by immunoassay kit (T4044T-100; Calbiotech, Inc) in duplicate determination.

Because blood sample volumes were limited by animal care regulations, we could not carry out all measurements at all time points. Therefore, only anti-TSHR autoantibody titers and  $T_4$  were measured at week 23, whereas only potency of autoantibodies to stimulate TSHR-dependent cAMP levels and  $T_4$  were measured at week 27. At the end of the experiment, additional measurement of anti-TSHR autoantibody titers by second generation assay was feasible for 44 out of 84 animals.

### Histological analysis

Histological methods are summarized in [Supplemental Materials and Methods](#).

### Statistics

Differences between the groups were analyzed by ANOVA for comparison between groups using SPSS software (version 19), followed by least significant difference (LSD) post hoc testing, or Student's  $t$  test where appropriate. For comparison of values at various times within 1 group, ANOVA for repeated measurements (RM-ANOVA) was used where appropriate.

Correlation coefficients were calculated according to Pearson.

## Results

### Animals and body weights

The body weights did not differ significantly between the groups during the study. At the end of the study, the

Ad-TSHR-immunized mice showed mean body weight gains of  $45 \pm 2.2\%$  ( $10^{10}$  pfu) and of  $45 \pm 2.4\%$  ( $10^{11}$  pfu) vs initial measurements, compared with  $50 \pm 3\%$  and to  $36 \pm 3\%$  in the respective GFP-immunized groups, reflecting normal growth of the animals during the study. Macroscopical investigation of the animals did not result in any abnormal observations of organs outside the thyroid and the heart.

### Anti-TSHR antibody titers and capacity to stimulate cAMP in test cells

Anti-TSHR antibodies were determined from serum samples. To this aim, 2 different ELISAs were used which detect the ability of the respective mouse sera to inhibit the binding of TSH or of the monoclonal Graves' patient antibody M22 to the TSHR. Both detected highly significant titers in animals which had received Ad-TSHR compared with the mock-immunized group.

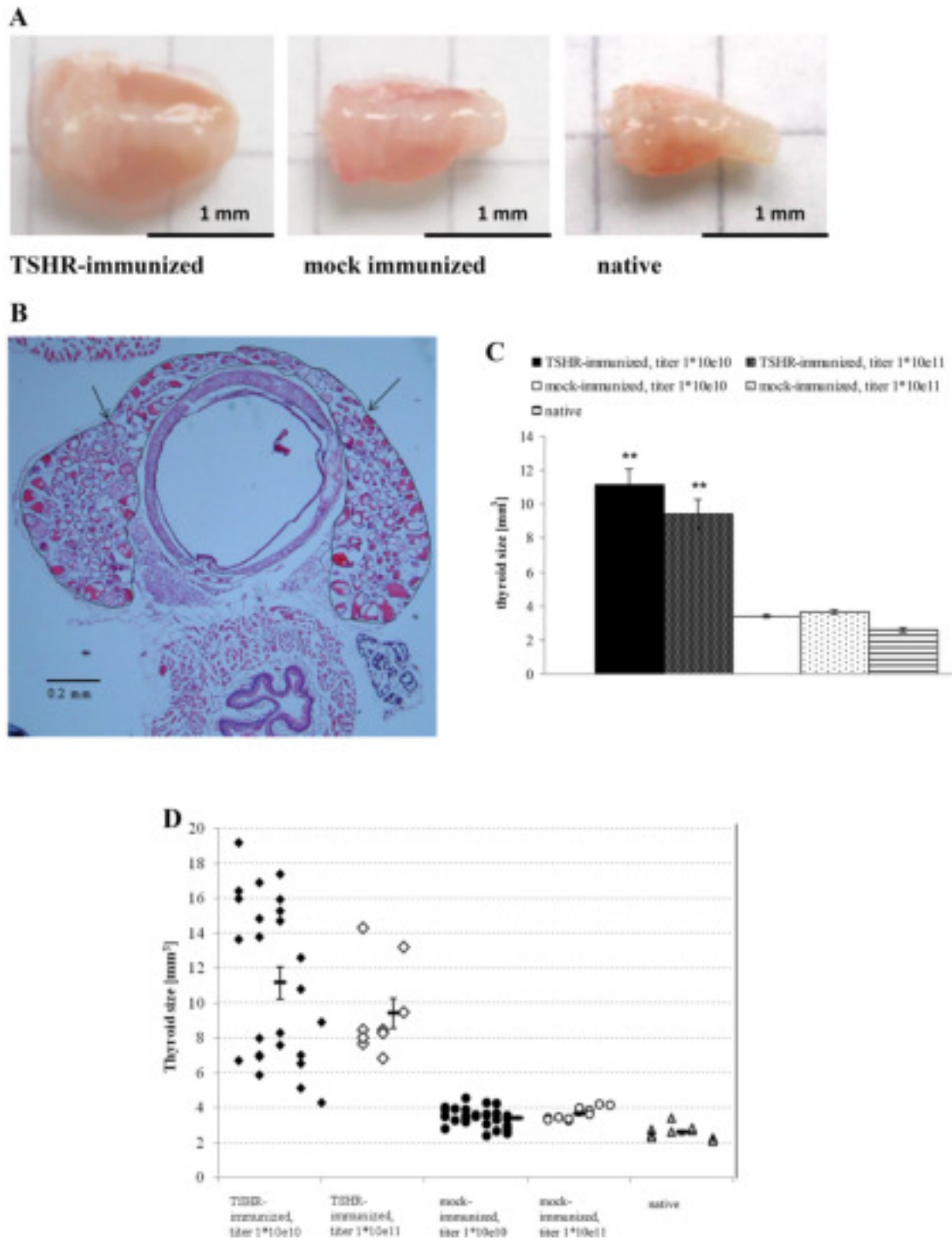
Mean anti-TSHR antibody titers increased progressively during the course of the study in both Ad-TSHR-immunized groups, as determined by third generation ELISA (Figure 2A). Looking at individual animals, it turned out that 100% of the Ad-TSHR-injected mice developed the expected anti-TSHR antibody titers during the experiment, albeit at a range of 10–900 IU/L. None of the mock infected controls showed any increase compared with the native group or basal values at any time. Figure 2B shows very comparable results obtained with a second generation assay at the end of the experiment.

In addition, the stimulatory activity of these antibodies was determined as the capacity of mouse serum samples to stimulate TSHR-dependent cAMP levels in test cells (Figure 3). This capacity was markedly and consistently increased throughout the study in serum samples taken from Ad-TSHR-immunized animals but was never detected in sera from the mock-immunized group.

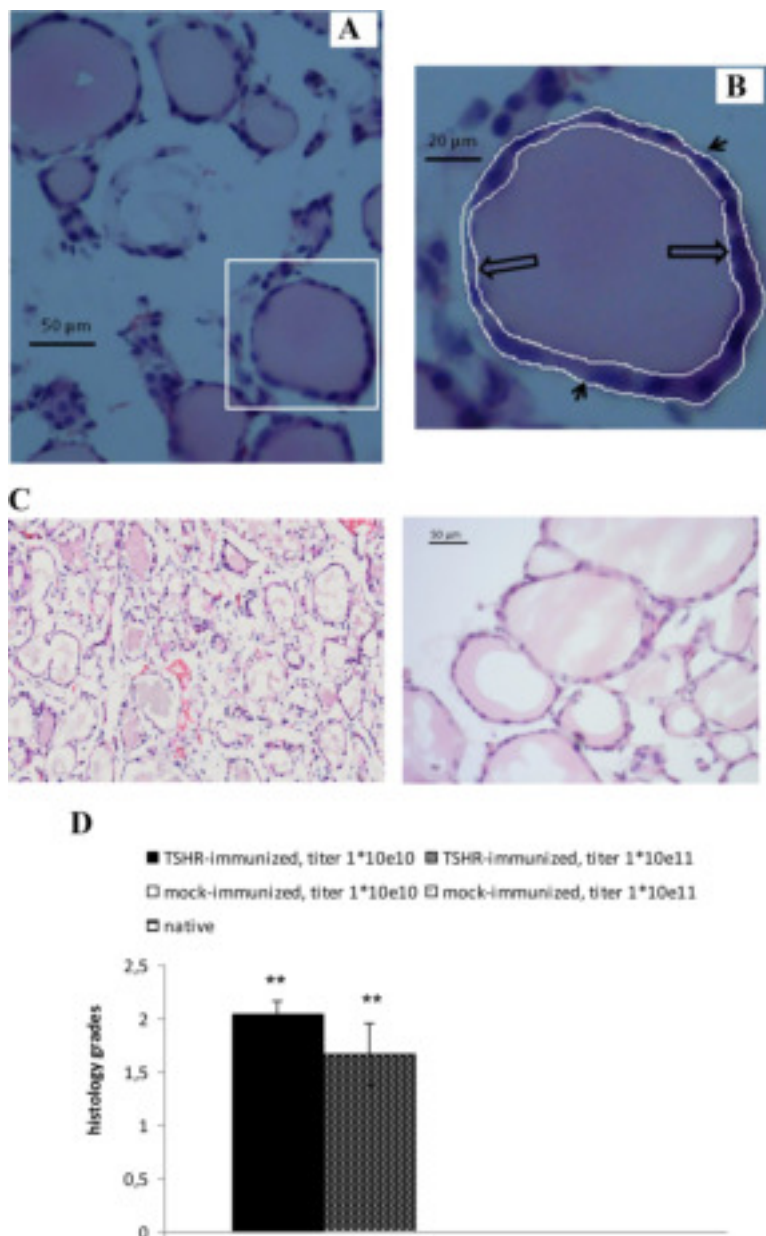
In addition, the stimulatory activity of these antibodies was determined as the capacity of mouse serum samples to stimulate TSHR-dependent cAMP levels in test cells (Figure 3). This capacity was markedly and consistently increased throughout the study in serum samples taken from Ad-TSHR-immunized animals but was never detected in sera from the mock-immunized group.

### Thyroid sizes, as determined macroscopically and from histological sections

Macroscopic investigation showed clearly increased thyroid sizes in mice that had received 9 immunizations of Ad-TSHR compared with mock immunizations. Figure 4A shows representative photographs.



**Figure 4.** Effect on thyroid size. A, Representative macroscopic images. The effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR on thyroid size. Comparison of the thyroid of a mouse immunized with 10<sup>10</sup> pfu Ad-TSHR (left) vs a thyroid from a mouse that was mock immunized (10<sup>10</sup> pfu Ad-GFP, middle panel) and a thyroid from a native mouse (right panel). Representative images are shown. B, Representative microscopic image of a cross-section of a thyroid. The indicated black line (arrows) encircles the thyroid tissue area. C, Effect on histologically measured thyroid size. Effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR was evaluated at the end of the main study. The mean thyroid size in mm<sup>3</sup> is shown with SEM. Difference between groups was tested by AVOVA followed by post hoc LSD testing. All measurements were carried out in 24 animals receiving 10<sup>10</sup> pfu Ad-TSHR, 9 animals receiving 10<sup>11</sup> Ad-TSHR, 32 animals receiving 10<sup>10</sup> pfu Ad-GFP, and 10 animals receiving 10<sup>11</sup> pfu Ad-GFP. In addition, age-matched immunologically naive mice (9 animals) were investigated. \*\*, Statistical significance (*P* < .001) compared with the mock-immunized group. D, Scatter chart of thyroid sizes. Results of individual measurements of thyroid sizes, including mean values (horizontal bars), are shown.



**Figure 5.** Qualitative histological investigation of thyroid sections. A and B, Representative microscopic image of a thyroid follicle. The endothelial lining cells and the inner colloid space can be readily distinguished at lower (A) or higher (B) magnification. C, Effect on histological grading of thyroid sections. The effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR on histological gradings (disease severity) was evaluated in microscopic thyroid sections. Comparison of a typical thyroid section of a mouse immunized with  $10^{10}$  pfu Ad-TSHR (left) vs a thyroid section from a mouse that was mock immunized ( $10^{10}$  pfu Ad-GFP, right panel). Representative images are shown. D, Effect on histological gradings of thyroid sections. Effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR, as evaluated at the end of the main study. The mean histological scores that were observed on thyroid sections are shown with SEM. Comparison of TSHR-immunized (left) vs mock-immunized (right panel) mice. Difference between groups was tested by ANOVA. All measurements were carried out in 14 animals (Ad-TSHR) and 30 animals (Ad-GFP). \*\*, Statistical significance ( $P < .001$ ) compared with the mock-immunized group. The mock-immunized and native groups all showed histological grades close to 0.

Histological investigation was carried out on all animals. Figure 4B shows a representative image of normal histology to show how thyroid size was measured.

Thyroid volumes ( $\text{mm}^3$ ) were determined from the sums of the areas of each section over the whole cutting regions (from 5 or 10 slides, depending on respective size of the thyroid gland) multiplied by the slice thickness of 0.5 mm. Thyroid sizes of the TSHR-immunized group showed a highly significant increase compared with the mock-immunized group (see Figure 4C). All Ad-TSHR-immunized animals developed increased thyroid size.

Figure 4D shows scatter plots of the individual results obtained in single animals.

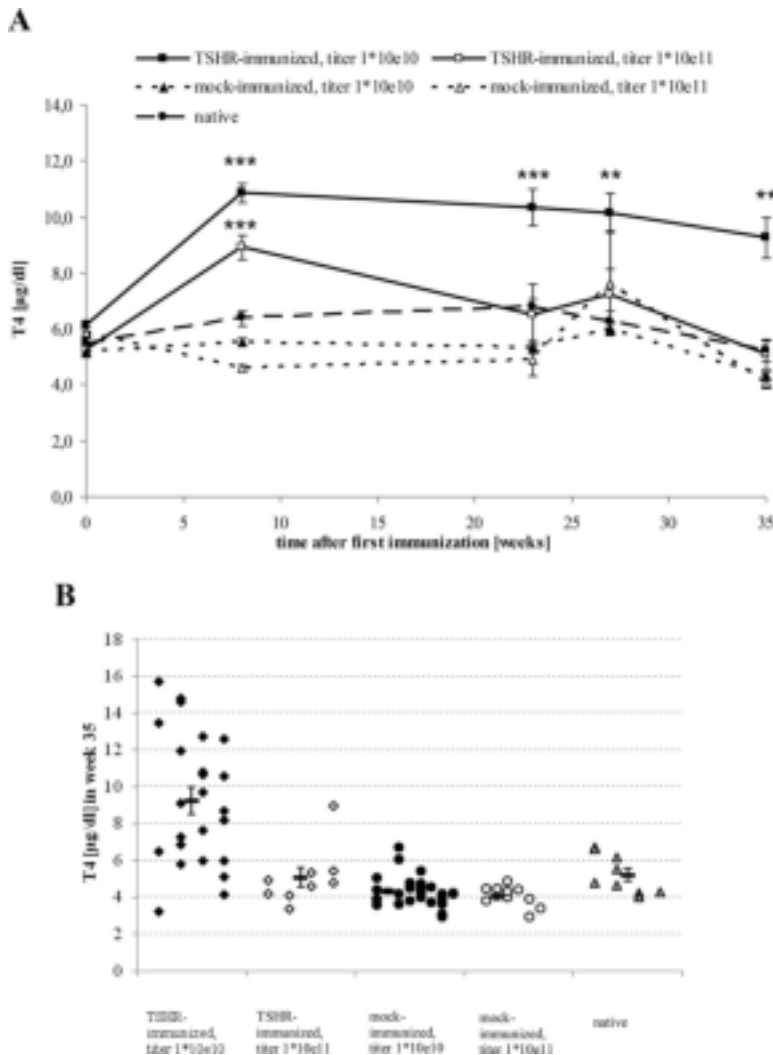
### Patho-histological changes

Also, a qualitative histological investigation was carried out on all animals. Figure 5, A and B, shows representative images of a normal thyroid histology to demonstrate how follicle size was measured.

In  $9 \times$  Ad-TSHR-treated mice, prominent infoldings of the hyperplastic follicular epithelium occurred, which led to fractioning of thyroid follicles, and corresponding smaller follicle and colloid sizes (Figure 5C, left panel). This degenerate histological image contrasted with the normal aspect of intact follicles and normal colloid size of mock-immunized animals (Figure 5C, right image). In summary, higher histological grades were frequently observed in TSHR-immunized animals, whereas mock-immunized mice did not show any pathology. Figure 5D shows a marked difference of mean histological scores between both groups.

### Pilot study of 7 immunizations over 20 weeks

Additionally, thyroid sizes were determined in a previous pilot study of mice, which received 7 consecutive Ad-TSHR or Ad-GFP immunizations at 3-weekly intervals over 20 weeks. The Ad-TSHR-immunized group showed a highly significant increase ( $P < .005$ ) in thyroid size ( $5.5 \pm 0.63$



**Figure 6.** Effect on serum T<sub>4</sub> levels. A, The effect of 9 immunizations with adenovirus encoding the A-subunit of the TSHR on serum T<sub>4</sub> levels was evaluated and compared with a mock-immunized group. Data are represented as mean ± SEM. All measurements were carried out in 24 animals receiving 10<sup>10</sup> pfu Ad-TSHR, 9 animals receiving 10<sup>11</sup> Ad-TSHR, 32 animals receiving 10<sup>10</sup> pfu Ad-GFP, and 10 animals receiving 10<sup>11</sup> pfu Ad-GFP. In addition, age-matched immunologically naive mice (9 animals) were investigated. Significance over time was tested by ANOVA of groups at given time points and controlled by RM-ANOVA within 1 group, followed by LSD post hoc testing. \*, *P* < .05; and \*\*, *P* < .005 and \*\*\*, *P* < .001, TSH-immunized group compared with the respective (titer-matched) mock-immunized group. B, Scatter chart of T<sub>4</sub> levels. Results of individual T<sub>4</sub> measurements at the end of the experiment, including mean values (horizontal bars), are shown.

mm<sup>3</sup>, *n* = 14) compared with the mock-immunized group (2.29 ± 0.19 mm<sup>3</sup>, *n* = 10), which did not differ compared with age-matched, nonimmunized healthy mice (2.70 ± 0.19 mm<sup>3</sup>, *n* = 6).

Histological investigation of the thyroids taken from these animals showed that the length of thyrocytes was increased in those animals which had been immunized with Ad-TSHR compared with Ad-GFP. The thyrocyte length was 5.64 µm ± 0.22 (Ad-TSHR, *n* = 14) compared with 3.61 µm ± 0.23 (Ad-GFP, *n* = 10) which did not differ significantly from the nonimmunized native age-matched group (3.52 µm ± 0.23, *n* = 6).

Histological evaluation of these TSHR-immunized animals of the pilot study rather showed cuboid epithelial cells and hypertrophied colloid follicles, corresponding to less progressive disease than that observed at the end of the main study. This lower disease severity was also reflected in an elevated, but less pronounced, mean histological score of 0.8 ± 0.15, whereas mean grading was 0 in mock-immunized mice and in native age-matched mice.

**Determination of T<sub>4</sub> serum levels**

T<sub>4</sub> levels did not differ between groups at study start. Mean T<sub>4</sub> levels in the group immunized with 10<sup>10</sup> pfu Ad-TSHR were consistently and significantly higher than controls throughout the further measurements of the study (Figure 6A). Compared with published normal values in mice (serum T<sub>4</sub> levels >8 µg/dL were considered hyperthyroid in BALB/c mice by most authors), T<sub>4</sub> levels were consistently increased beyond that cut-off value in the group immunized with 10<sup>10</sup> pfu Ad-TSHR. In contrast, T<sub>4</sub> levels tended to decrease after peaking initially in the group immunized with 10<sup>11</sup> pfu Ad-TSHR.

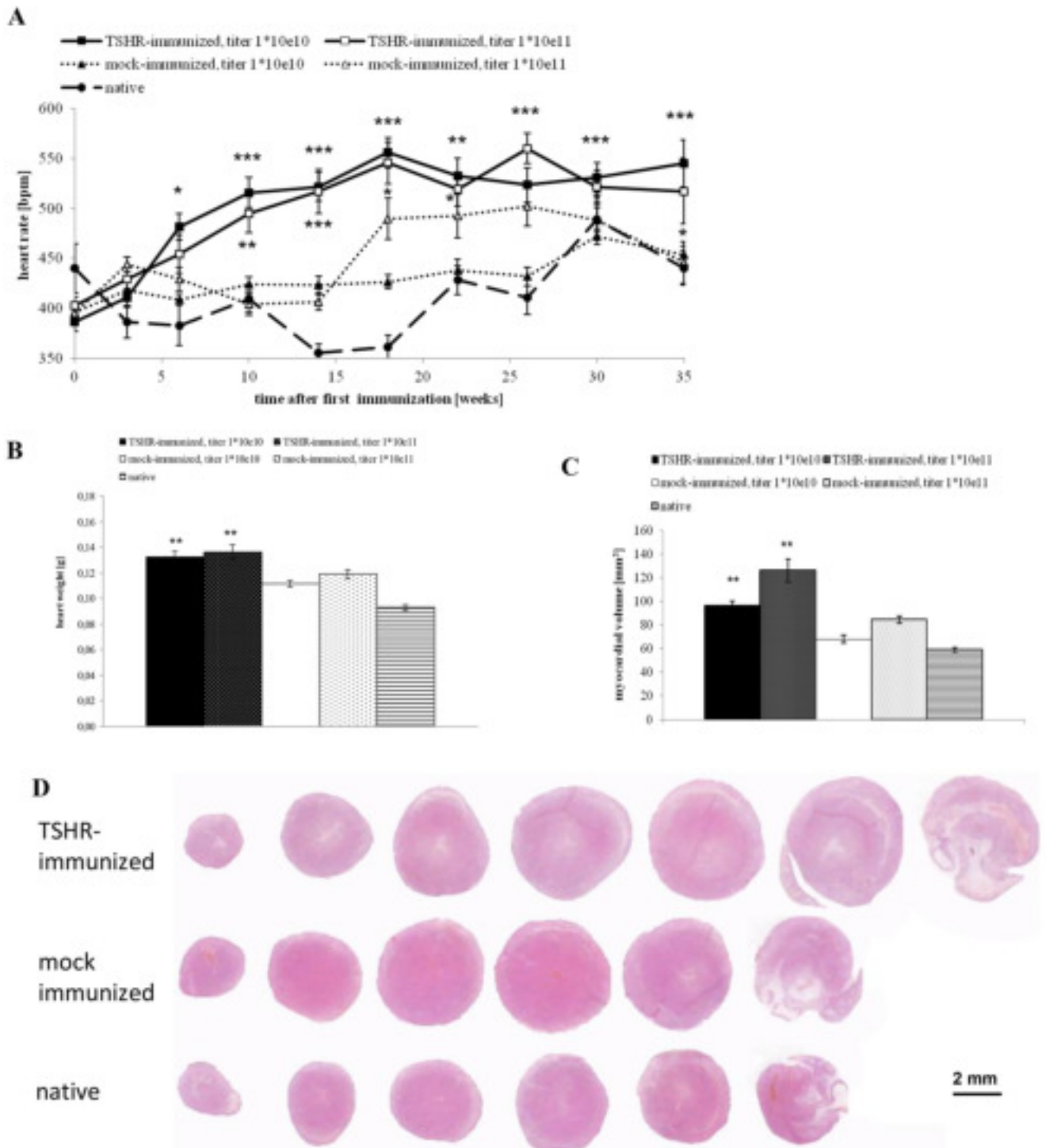
Figure 6B shows scatter plots of individual results obtained in single animals, thus demonstrating that results were fairly homogenous in single groups.

**ECG to determine heart rates**

Starting from the third immunization on (42 d after first immunization), a significant increase in heart rate in the hyperthyroid group immunized with 10<sup>10</sup> pfu Ad-TSHR was observed (to 121 ± 3% of heart rate at start) (Figure 7A). In contrast, heart rate in the mock-immunized group did not change significantly (eg, 10<sup>10</sup> pfu Ad-GFP to 105 ± 2% of heart rate at start).

There was a further strong increase in heart rate in the hyperthyroid group immunized with 10<sup>10</sup> pfu Ad-TSHR until the sixth immunization (to 142 ± 4%). This increase was highly significant for the group immunized with 10<sup>10</sup> pfu Ad-TSHR compared with the respective mock-immu-





**Figure 7.** Effect on heart rates (A), heart weights (B), and ventricular volumes (C and D). The effects of 9 immunizations with adenovirus encoding the A-subunit of the TSHR on heart rates at various times during the experiment (A), and on heart weights (B) and cardiac ventricular volumes (C and D) at the end of the experiment, were evaluated and compared with a mock-immunized (Ad-GFP) group. Data are represented as mean  $\pm$  SEM. All measurements were carried out in 24 animals receiving  $10^{10}$  pfu Ad-TSHR, 9 animals receiving  $10^{11}$  Ad-TSHR, 32 animals receiving  $10^{10}$  pfu Ad-GFP, and 10 animals receiving  $10^{11}$  pfu Ad-GFP. In addition, age-matched immunologically naive mice (9 animals) were investigated. Significance over time was tested by ANOVA of groups at given time points and controlled by RM-ANOVA within 1 group, followed by LSD post hoc testing. A, \*,  $P < .05$ ; \*\*,  $P < .005$ ; \*\*\*,  $P < .001$ , TSH-immunized group compared with the respective (titer-matched) mock-immunized group. B, \*\* indicates statistical significance ( $P < .001$ ) compared with the mock-immunized group. C, \*\* indicates statistical significance ( $P < .001$ ) compared with the mock-immunized group. D, Comparison of the myocardium of a mouse immunized with  $10^{10}$  pfu Ad-TSHR (upper panel) vs myocardium from a mouse that was mock immunized ( $10^{10}$  pfu Ad-GFP, middle panel) and myocardium from a native mouse (lower panel). Representative images are shown.

nized group ( $10^{10}$  pfu Ad-GFP; increase to  $116 \pm 3\%$ ). The group immunized with  $10^{11}$  pfu Ad-GFP showed a relevant increase in resting heart rate by its own, probably due to an immune response to the high virus titer, so that the difference between heart rates observed after immunization with  $10^{11}$  pfu Ad-TSHR and  $10^{11}$  pfu Ad-GFP was not significant at all time points. Most of the evaluated ECGs related to sinus tachycardia, small stretches of arrhythmia occurred, but could not be unequivocally related to AF, perhaps owing to registration quality.

### Heart weights

Macroscopical investigation and preparation of mouse hearts upon necropsy revealed significantly increased heart weights in the Ad-TSHR-immunized groups (Figure 7B). Additionally, calculation of myocardial volumes by adding up digitized cross-sectional LV areas from 6 consecutive sections, which were evenly distributed across the fixated hearts, showed increased cardiac ventricular volumes (Figure 7C).

Histological assessment of 10 representative hearts in each group revealed signs of cardiomyocyte hypertrophy and cardiomyopathy. These included increases in cell size and thicker myocardial fibers in the hematoxylin eosin-stained sections of the Ad-TSHR-immunized animals hematoxylin eosin. There were no round cell infiltration, vacuolar degeneration of myofibers, atrophic myofibers, lipid deposits, or cords of collagen found in animals from the TSHR groups. Therefore, the cardiac enlargement was due to tissue hypertrophy and not to edema.

### Correlations

Correlations were determined for all end-of-study results of all animals that were investigated in the main study (Table 1). It can be seen that high correlations coefficients more than 0.7 were observed for thyroid sizes vs  $T_4$  levels, vs anti-TSHR titer levels, and vs heart weights and heart rates. In contrast, thyroid sizes did not correlate as well with the absolute cAMP-stimulatory capacities of the anti-

TSH antibodies on test cells (“cAMP”), which were generally not well correlated to the other parameters.

### Discussion

In this study, we show that adenovirally induced TSHR A-subunit immunization in mice can be extended by using a novel protocol, in which regular 4-weekly injections serve to continuously boost antibody production. Using this protocol, generation of TSH binding antibodies is induced, which show persistent capacity to stimulate cAMP activity in TSHR-expressing test cells. Similarly, histological investigation showed goiter and follicular thyroid hyperplasia in long-term immunized animals for at least 9 months. Elevated  $T_4$  levels were observed in the group immunized with  $10^{10}$  pfu Ad-TSHR. Moreover, cardiac involvement (tachycardia and cardiac hypertrophy) were consistently observed in all Ad-TSHR-immunized mice.

Previous studies have described the establishment of Graves’ phenotype in TSHR-immunized mice. This was true for the transfected fibroblast model (24), in which about 25% of injected mice developed hyperthyroidism (as determined by  $T_4$  levels), and for recombinant dendritic cell injections (25). After some failed efforts to use plasmid immunizations, adenovirally induced immunization in balb/c mice was successfully established to induce a mouse model of Graves’ disease (9, 13). The use of adenovirus was more effective than use of plasmid transfection in most studies, with the exception of a novel protocol of electroporation (15, 16), which holds promise to investigate orbital fibrosis in these mice. This latter protocol consisted of 4 3-weekly immunizations over a total period of 3 months. After plasmid electroporation, long-term expression of stimulatory anti-TSHR antibodies and persistent thyroid enlargement had been described (17). However, this disease model seems to have caused a relevant mortality of the investigated mice starting 4 months after the last electroporation (17).

**Table 1.** Correlation Coefficients of Results

Correlation Coefficient R	Thyroid Size	Heart Weight	Heart Rate	$T_4$	Third Generation Assay	cAMP
Thyroid size	—	0.7660	0.7144	0.8427	0.7029	0.5168
Heart weight		—	0.5501	0.5251	0.4985	0.4433
Heart rate			—	0.6626	0.5064	0.4144
$T_4$				—	0.7151	0.3015
Third generation assay					—	0.1924
cAMP						—

Pearson’s correlation coefficients R were determined for the results of all measurements in all 84 animals (comprising 24 animals receiving  $10^{10}$  pfu Ad-TSHR, in 9 animals receiving  $10^{11}$  Ad-TSHR, in 32 animals receiving  $10^{10}$  pfu Ad-GFP, 10 animals receiving  $10^{11}$  pfu Ad-GFP, and 9 age-matched immunologically naive mice). We compared the values obtained at 35 weeks (final measurements). Parameters were thyroid sizes as determined from histological sections,  $T_4$  levels, anti-TSHR titer levels (third generation assay), heart weights, heart rates, and absolute cAMP-stimulatory capacities of anti-TSH antibodies on test cells (cAMP levels measured as pmol/mL, cAMP).

The establishment of various protocols to induce Graves' disease led to the challenge of comparing these models, because individual features differed between them (13). Elevated  $T_4$  levels, stimulating anti-TSH antibodies, goiter, and typical histological alterations were considered to be essential characteristics of any model to define relevance for human disease (13). In these mouse models, TSHR antibodies were detectable by direct binding to ELISA wells coated with TSHR A-subunit protein purified from CHO cells (eg, 10, 12). These studies of induced TSHR antibodies and hyperthyroidism also employed assays that measured inhibition of TSH binding and/or cAMP generation from TSHR-expressing eukaryotic cells. These features were all also observed in the current long-term study.

Long-term persistence of the models using adenoviral gene transfer has not yet been clearly described. One study on a prolonged observation period of 14 weeks after 3 adenoviral TSHR A-subunit immunizations over 6 weeks (total duration 20 wk) showed that anti-TSHR antibody titers were consistently detected until the final evaluation but that their potency to stimulate cAMP had considerably receded at that time, and no  $T_4$  elevation nor thyrocyte hyperplasia was observed at the end of the study (18). Regulatory T-cell depletion and adjuvant in TSHR transgenic mice did not reliably induce Graves' disease (26).

In contrast to these approaches, we sought to study an alternative to a maximum of 3 immunizations and/or a 3-month immunization period, ie, to maintain immunizations at regular intervals for many more months. We chose 4-weekly injections for this maintenance period, in accordance with Jahns et al (19). We found that in partial contrast to the previous approaches, the disease phenotype was stably induced by this protocol.

Anti-TSHR antibody titers were consistently measured with 3 different assays. These assays included the current gold standard "third generation" immunoassay, which detects the ability of the respective mouse sera to inhibit the binding of the monoclonal Graves' patient antibody M22 to the TSHR (RSR-Cobas Roche), which is most often used to identify Graves' disease in humans. This assay was reported to identify Graves' patients with a specificity and sensitivity of more than 97% (27, 28). To our knowledge, our study is the first to report that the functionally active anti-TSHR antibodies observed in immunized mice are readily detected by this same assay. Use of identical assays also allowed to compare absolute titer values. The range of anti-TSHR titers in immunized mice was higher than that typically found in Graves' patients, in accordance with previous studies and judgments (12, 18). Rather surprisingly, however, our direct comparison based on absolute values showed that the range measured

in the animals by the M22 inhibition assay (10–900 U/L; mean, 132 U/L) was still in the same logarithmic order of magnitude than the titers observed in human Graves' disease patients (typically, ~1.5–100 U/L). This is in contrast to animal models of other autoimmune diseases, in which titers were several log orders of magnitude higher than in the respective patients.

Previous published titer measurements and studies, eg, on the distinction of higher vs lower titers (12, 18), did not include absolute titer values, and study results were often expressed as inhibition percentage of TSH binding (29). We think that our measurements with the M22-based RSR third generation assay insofar offer an interesting complementation to these previous studies. All TSHR-immunized animals developed such an anti-TSHR antibody titer measurable by this RSR third generation assay, but none of the mock-immunized control mice did. This shows the potency of immunizing native balb/c mice and is a superior efficacy percentage compared with some previous approaches.

In the same animals, we found consistent and marked thyroid hyperplasia. Histological features included increased thyrocyte length and cuboid epitheloid hyperplasia after 7 months (pilot study), and a degenerate image with prominent infoldings of follicles, smaller follicle size, and vacuolization at 9 months (main study).

Mean  $T_4$  values were elevated after immunization with  $10^{10}$  pfu Ad-TSHR. In contrast, higher titers ( $10^{11}$  pfu Ad-TSHR) led to an initial  $T_4$  peak, and  $T_4$  levels then tended to decline at further measurements and were observed in the range of the values of the control groups. This is partly in accordance with previous studies (10). Actually, it might be due to the immune response to the higher virus titer of  $10^{11}$  pfu. Unfortunately, we could not further clarify this issue.

Graves' disease results in increased morbidity and mortality (30), mainly due to cardiac complications (2). Tachycardia is a reliable marker of disease severity in hyperthyroid patients (2, 3). AF occurs in 5%–15% of patients (2). Twenty-four-hour ECG monitoring showed that heart rate is constantly increased during the day (3). Therefore, we sought to investigate the clinically important cardiac involvement also in this animal model. ECG registrations served to detect the effect on heart rate. Tachycardia developed progressively and was significantly and stably observed throughout months 3–9 of the study in TSHR-immunized animals compared with mock-immunized controls. Although mostly sinus tachycardia was observed, smaller stretches of AF with concomitant absolute arrhythmia could not be excluded. ECG registration allowed to detect QRS and RR intervals with certainty, but quality did not suffice to image baseline and P

waves securely. Thus, this interesting question must be referred to future studies with higher ECG power. A small increase of heart rate also occurred in these control animals compared with baseline. The ECG finding corroborate a recently published report on Ad-TSHR immunization in rhesus macaque monkeys, in which 3 animals were successfully immunized with 7 consecutive injections over 20 weeks (31). Clinical investigation of these 3 animals also indicated increased heart rate (no details on method were given).

Also, investigation of orbital histology or function would be an additional interesting measurement. It has been shown to be altered in previous studies using electroporation and plasmid gene transfer (16). Unfortunately, we could not investigate this issue within the current study, but we will try to include these parameters in future studies of the same model.

Correlations coefficients for results of individual animals at the end of the study reached fairly high values for thyroid sizes vs serum T<sub>4</sub> levels, vs anti-TSHR titer levels and vs heart weights and heart rates. In contrast, the absolute cAMP-stimulatory capacity (determined as pmol/mL cAMP, which was generated in the test cells) of the anti-TSH antibodies (cAMP) was generally less correlated to the other parameters.

This finding indicates that the animal model is consistently characterized by generation of specific anti-TSHR antibodies whose levels in single animals impact on the amount of thyroid enlargement, and on serum T<sub>4</sub> levels as well as on the cardiac consequences of disease. In contrast, we would speculate that effects of anti-TSHR antibodies on target cells beside cAMP stimulation should also be relevant, because the biological effect of these antibodies is not precisely predicted just by measuring their capacity to stimulate cAMP levels in test cells. Such alternative second messenger systems could depend on stimulation of G<sub>q</sub> and consequent activation of phospholipase C or other intracellular enzymes but might also imply activation of G<sub>i</sub>-dependent pathways. This interesting question should be investigated in future studies, in which the effect of anti-TSHR antibodies on other intracellular second messengers than cAMP should be determined. However, the finding may also possibly be explained by lesser cross-reactivity of the antihuman TSHR antibodies generated in this model for the natively occurring mouse TSHR.

In summary, the study provides a stable model of human Graves' disease and of the cardiac involvement in hyperthyroidism.

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