

# Experimental Reproduction of Dynamic Fluctuation of TSH Receptor–Binding Antibodies Between Stimulation and Inhibition

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**Context:** Hyperthyroidism in Graves disease (GD) is caused by autoantibody stimulation of the TSH receptor (TSHR). TSHR autoantibody (TSHR-Ab) activity is measured routinely by inhibition of labeled ligand (TSH or M22) binding to the TSHR [TSH-binding inhibitory immunoglobulins (TBIs)] or by stimulation of cAMP production in isolated cells [TSH receptor–stimulating antibodies (TSAs)]. Usually, measurements of TSHR-Abs by TBIs agree reasonably well with TSA values at least in the setting of hyperthyroidism, and both measurements tend to change in parallel during treatment with some exceptions. In this study, we describe three unusual cases, which illustrate nearly pure stimulating, blocking, or neutral properties of TSHR-Abs.

**Objective:** Whether patient serum TSHR-Abs can be reproduced by mixtures of human monoclonal autoantibodies to the TSHR was studied because the sera in most patients show moderate properties having both of TBII and TSA activities.

**Design:** We compared the TBII and TSA activities of serum from four unusual patients in detail with mixtures of human monoclonal TSHR-Abs (mAbs) M22 (stimulating), K1-18 (stimulating), and K1-70 (blocking).

**Results:** Characteristic of a patient's serum was similar to M22 or K1-18, another was similar to K1-70, whereas another was similar to a mixture of K1-70 and M22 (or K1-18). Additionally, some patients seemed to have neutral TSHR-Abs in their sera.

**Conclusions:** Our studies suggest that the characteristics of TSHR-Abs in the patient serum can be mimicked by mixtures of human mAbs to the TSHR, stimulating, blocking, and neutral if any.

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**Freeform/Key Words:** TSH receptor-stimulating antibodies, TSH receptor-blocking antibodies, TSH-binding inhibitory immunoglobulins

Abbreviations: AITD, autoimmune thyroid diseases; ATD, anti-thyroid drug; bTSH, bovine TSH; EIA, enzyme immunoassay; FT3, free T3; FT4, free T4; GD, Graves disease; mAb, monoclonal antibody; TBAb, TSH receptor–blocking antibody; TBII, TSH-binding inhibitory immunoglobulin; TgAb, thyroglobulin autoantibody; TPOAb, thyroid peroxidase autoantibody; TRAb, TSH receptor antibody; TSA, TSH receptor–stimulating antibody; TSHR, TSH receptor; TSHR-Ab, TSH receptor autoantibody.

Graves disease (GD) and Hashimoto thyroiditis are the most frequently encountered autoimmune thyroid diseases (AITDs). Hyperthyroidism in GD is caused by stimulating TSH receptor (TSHR) autoantibodies (TSHR-Abs) [1]. Assays for TSHR-Abs used clinically are usually based on inhibition of labeled ligand (TSH or M22) binding to the TSHR [thyroid-binding inhibitory immunoglobulins (TBIs)] or stimulation of cAMP production in isolated cells [TSH receptor–stimulating antibodies (TSAbs)]. TBI assays measure the sum of stimulating and blocking TSHR-Abs [2]. The blocking activity (TBAs) is defined as the inhibition of TSH stimulation on TSHR and is not an inhibition *per se*, so some TBAs can be a weak stimulator in the absence of TSH. Usually, TSHR-Abs in GD have stimulating activity. However, in some patients both stimulating and blocking TSHR-Abs coexist. The clinical condition, that is, hyperthyroidism or hypothyroidism, is the consequence of the sum of action of a polyclonal population of TSHR-Abs with different concentration, affinity, and stimulating/blocking activities. In fact, the stimulating monoclonal antibody (mAb) K1-18 and the blocking mAb K1-70 were isolated from a single patient at our clinic [3]. In this study, we investigated the discrepancies that are often observed in the detection of TSHR-Abs between binding assays and bioassays in various clinical views of AITDs. By mixing TSHR mAbs of known functional activity obtained from patients' lymphocytes (*i.e.*, K1-18, K1-70, and M22 [4, 5]), we have tried to experimentally reproduce the physiological changes in TSHR-Abs occurring in four peculiar patients from our clinic. This indicates that TSHR-Abs in the serum of GD patients can be mimicked by mixtures of various proportions of stimulating and blocking TSHR mAbs and some neutral Abs [6], and that the discrepancies between binding assay and bioassay may derive from the difference in their composition.

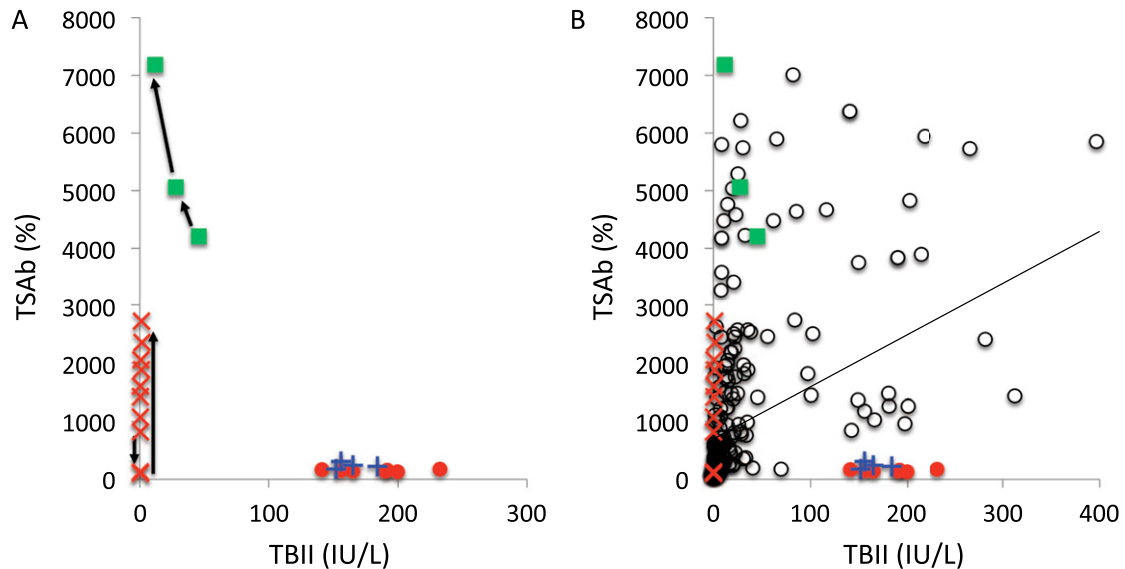
## 1. Patients and Methods

### A. Patients

Patient K was described elsewhere [3]. Briefly, she was a 52-year-old woman with hypothyroidism, having high TBI but low TAb values. She had an 8-year history of AITD and first presented with hyperthyroidism, was successfully treated with thiamazole for 3 years, but then developed hypothyroidism and was treated with levothyroxine. She had a small goiter but no signs of ophthalmopathy. Her TBIs varied between 152 and 184 IU/L and her TAb values varied between 180% and 309% for 2 years (blue plus signs in Fig. 1). In contrast, her TBA activities were highly positive (98.6% to 99.3%). After the publication [3], patient K has changed to be in a hyperthyroid state again and treated with thiamazole at the time we prepared this manuscript (recent TBIs and TAb values were 156 to 197 IU/L and 957% to 1260%, respectively).

We have experience with three other cases of GD, which help to elucidate the clinical significance of different types of TSHR-Abs. An 80-year-old man (patient M) visited our hospital with a complaint of impaired vision. He was initially found to have subclinical hyperthyroidism: 3.35 pg/mL free T3 (FT3), 1.09 ng/dL free T4 (FT4), and <0.003 mU/L TSH. Thyroglobulin autoantibodies (TgAbs) and thyroid peroxidase autoantibodies (TPOAbs) were both positive: >4000 IU/mL and > 600 IU/mL, respectively. Although the second-generation TBIs measured using DYNtest TSH receptor antibodies (TRAbs) (human) and the third-generation TBIs measured using ECLusys TRAbs were both undetectable (<1.0 IU/L and <0.30 IU/L, respectively), his initial TAb level was as high as 817%. He was diagnosed with Graves orbitopathy and treated with IV methylprednisolone (500 mg three times per week for 3 weeks) and radiotherapy (20 Gy in 10 daily doses of 2 Gy during 2 weeks) followed by daily doses of 40 mg of prednisolone, which was tapered. Then, the TAb values gradually decreased to 105%, but increased again up to 2717% (TBIs were 1.18 IU/L by ECLusys and 1.7 IU/L by DYNtest at this time) in a year after stopping the prednisolone (red × in Fig. 1).

A second patient (patient S, a 52-year-old female), without thyroid-associated orbitopathy, was referred to our clinic. She had hypothyroidism and was treated with 100 µg of



**Figure 1.** Relationship between TBII and TSAb in the patients with GD. (A) TSAb values were plotted against TBII values in the sera at the different time points of patients K (+, from March 2006 to July 2008) and T (green squares, from February 2007 to May 2007) measured by a DYNOTest TRAb assay based on inhibition of  $^{125}\text{I}$ -TSH binding and patients M ( $\times$ , from February 2017 to October 2018) and S (red circles, from July 2016 to December 2018) measured by ECLusys TRAb assay based on inhibition of M22 binding. Arrows indicate the chronological change in the data (see text). (B) TSAb values were plotted against TBII at all time points from 162 patients with GD (open circles) superimposed on (A), who are untreated or treated with ATDs, as shown in Table 1. The approximate line for all GD patients was drawn using Microsoft Excel<sup>®</sup>.

levothyroxine. Her TgAb and TPOAb levels were both positive: 50 IU/mL and 600 IU/mL, respectively. Her TBII varied between 141 and 233 IU/L, and her TSAb varied between 131% and 204% (red circles in Fig. 1). However, in contrast to patient K, her TBAb activity was negative ( $-26.3\%$  to  $0.9\%$ ; red circles in Fig. 3B), suggesting that her TSHR-Abs are almost neutral, with a slight TSAb activity, and her hypothyroidism is due to Hashimoto thyroiditis.

A third patient (patient T, a 58-year-old female) with thyroid-associated orbitopathy attended at our clinic. She had hypothyroidism with 0.87 ng/dL FT4, 2.60 pg/mL FT3, and 29.7 mU/L TSH. Her TgAb and TPOAb levels were both positive: 178 IU/mL and 295 IU/mL, respectively. Her TBII level was 45.3 IU/L and her TSAb level was 4210%. TSAb increased to 7180% whereas TBII decreased to 11.8 IU/L in 3 months after steroid pulse therapy (green squares in Fig. 1).

In summary, patient K was hypothyroid without thyroid-associated orbitopathy, with high TSHR-Ab levels by TBII and highly positive TBAb activity but low TSAb activity. Patient M presented with subclinical hyperthyroidism, thyroid-associated orbitopathy, and had high serum TSAb activity but not detectable by TBII. Patient S was also hypothyroid without thyroid-associated orbitopathy, with high TSHR-Ab levels by TBII, low serum TSAb activity but negative TBAb activity. Patient T was hypothyroid and had thyroid-associated orbitopathy, with high levels of serum TSHR-Abs by TBII and by bioassay. Additionally, her TSAb values decreased with increasing values of TBII by the steroid pulse therapy.

### B. Laboratory Evaluation

Serum concentrations of FT3, FT4, and TSH were determined by chemiluminescence immunoassay (Abbott Japan, Chiba, Japan). The reference ranges were 1.71 to 3.71 pg/mL, 0.70 to 1.48 ng/dL, and 0.35 to 4.94 mU/L, respectively. Anti-TgAbs and anti-TPOAbs were

measured with an electrochemiluminescence immunoassay (Roche Diagnostics, Tokyo, Japan). The reference ranges were <27 IU/mL and <15 IU/mL, respectively. TSHR-Abs were measured with an electrochemiluminescence immunoassay based on inhibition of M22 binding (ECLusys, TRAb M22, Roche Diagnostics) [7, 8] and with an assay based on inhibition of  $^{125}\text{I}$ -TSH binding [DYNTest TRAb (human), BRAHMS, Berlin, Germany] [9]. Results of both TBII assays are reported in international units per liter based on a World Health Organization standard, and the normal range is <2.0 IU/L for ECLusys and <1.0 IU/L for the DYNTest. The upper limits of detection of ECLusys and DYNTest were both 40 IU/L, and levels >40 IU/L were remeasured by diluting with pooling sera obtained from healthy blood donors. Thyroid stimulating activity (TSAbs) was assessed using a bioassay based on stimulation of cAMP production by porcine thyroid cells [TSAb enzyme immunoassay (EIA) kit, Yamasa Corporation, Chiba, Japan] [10, 11]. TSHR blocking activities (TBAbs) were assessed using a modified TSAb EIA kit [12]. Briefly, 75  $\mu\text{L}$  of serum was treated with 25  $\mu\text{L}$  of dextran-coated charcoal to absorb endogenous cAMP. Then, 25  $\mu\text{L}$  of treated sample (in duplicate) was mixed with 50  $\mu\text{L}$  of reaction buffer (provided in the TSAb EIA kit) in the presence or absence of 100 mU/L of bovine TSH (bTSH), and 50  $\mu\text{L}$  of porcine thyroid cell suspension (provided in the TSAb EIA kit) was added to each well. The plate was incubated at 37°C for 4 hours and then 100  $\mu\text{L}$  of lysis buffer (provided in the TSAb EIA kit) was added to each well. The cAMP concentration in the lysed porcine thyroid cells was then measured by ELISA. Negative TSAb control was prepared by pooling sera obtained from healthy blood donors. Positive TSAb control was prepared by pooling sera obtained from GD patients. For concentration tests, IgG was purified from the patient's sera using Pierce Thiophilic Adsorbent and Purification Kit (Thermo Fisher Scientific, Tokyo, Japan) and concentrated using Amicon Ultra 0.5-mL centrifugal filters (molecular mass cutoff of 50 kDa; Merck, Darmstadt, Germany).

### C. Characterization of mAbs

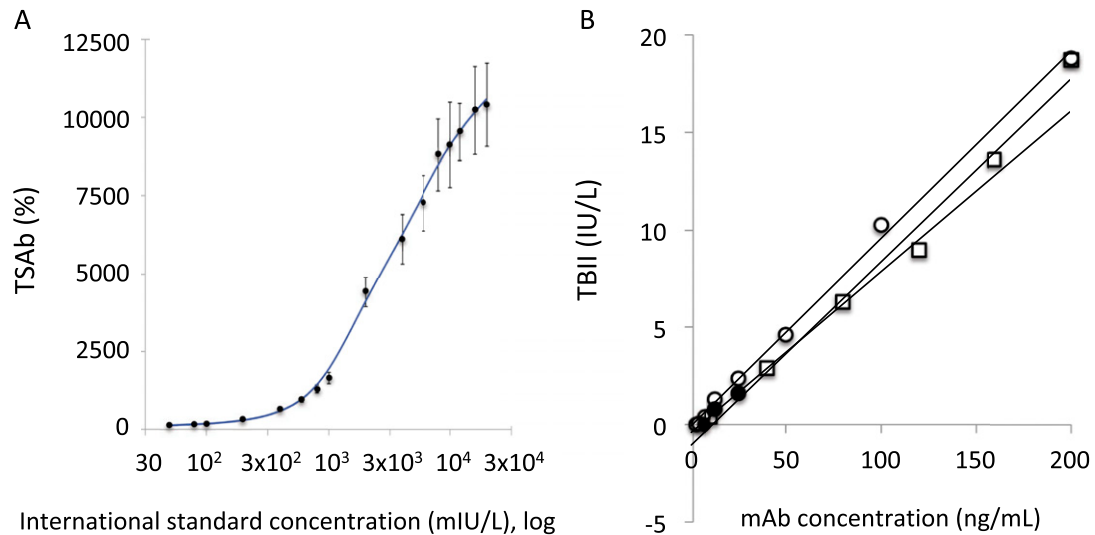
M22 [13], K1-18 [14], and K1-70 [15] mAbs were provided by Dr. B. R. Smith at FIRS Laboratories, RSR Ltd. (Cardiff, UK). Different concentrations of M22, K1-18, K1-70, and the second international standard for TSAb (National Institute for Biological Standards and Control code 08/204, 113 mIU per ampoule) [16] were diluted in healthy blood donor serum and assayed for TBII, TSAb, and TBAbs. The dose response curve of the second international standard for TSAb is shown in Fig. 2A. The dose response curves of three kinds of mAbs for TBII (by DYNTest) against their concentrations are shown in Fig. 2B. The relationship between mAb concentration and TBII value was linear, and no difference was observed among mAb species, especially between stimulating and blocking mAbs. The dose response curve of K1-70 for TBAbs against its concentration and TBII are shown in Fig. 3A and 3B, respectively.

TSAb values were calculated as follows:  $\text{TSAb (\%)} = [100 + (s - n)/(p - n)] \times 650$ . The variables are defined as:  $n$ , cAMP produced by negative control serum (TSAb = 100) provided in the kit;  $p$ , cAMP produced by positive control serum (TSAb = 750) provided in the kit; and  $s$ , cAMP produced by patient serum. The normal range of TSAb is <120% according to the package insert.

TBAb values were calculated as follows:  $\text{TBAb (\%)} = [1 - (c - d)/(a - b)] \times 100$ . The variables are defined as:  $a$ , cAMP produced by 100 mU/L bTSH and negative control serum provided in the TSAb EIA kit;  $b$ , cAMP produced by negative control serum provided in the TSAb EIA kit;  $c$ , cAMP produced by 100 mU/L bTSH and patient serum; and  $d$ , cAMP produced by patient serum. The normal range of TBAbs is <34% [17].

### D. Statistical Analysis

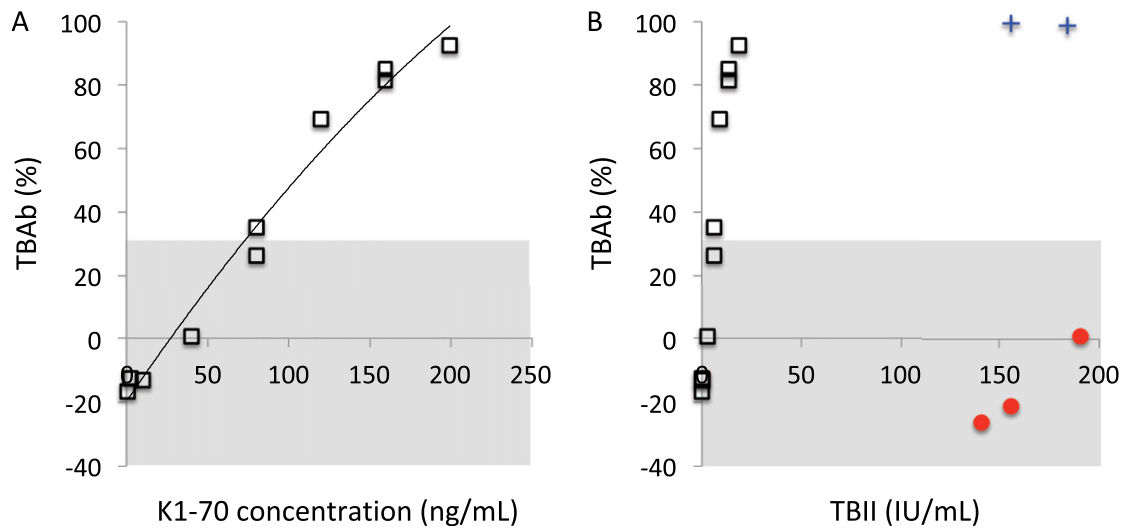
Statistical analysis was performed using the Statistical Package for Social Sciences version 24 (SPSS, Chicago, IL). Normally distributed continuous variables were shown as mean values with SD (mean  $\pm$  SD). A Spearman rank correlation coefficient was used to evaluate the correlation.



**Figure 2.** Dose response curve of the second international standard for TSABs. (A) Various concentrations of the international standard (National Institute for Biological Standards and Control code 08/204) are plotted on a logarithmic scale on the *x*-axis against the corresponding TSAb values on the *y*-axis. Mean  $\pm$  SD of quadruplicate measurements for each standard concentration is shown. The polynomial approximate curve was drawn using Microsoft Excel<sup>®</sup>. (B) Various concentrations of M22 (●), K1-18 (○), and K1-70 (□) mAbs are plotted against the corresponding TBII values measured by DYNtest TRAb assay based on inhibition of <sup>125</sup>I-TSH binding. The linear approximate curves were drawn using Microsoft Excel<sup>®</sup>.

## 2. Results

Because of large discrepancies between the TBII and TSAb activities, especially in patients M and K, we compared them with other GD patients to clarify the incidence of this phenomenon. Institutional Ethics Committee approval for the study was obtained and, after obtaining



**Figure 3.** Dose response curve of K1-70 for TBABs. (A) TBAB values in various concentrations of K1-70 are plotted. (B) The same TBAB values to (A) in various concentrations of K1-70 are replotted against the corresponding TBII values measured by a DYNtest TRAb assay based on inhibition of <sup>125</sup>I-TSH binding and they were superimposed on the TBAB data of patients K (+) and S (red circles). A shadow indicates the reference (negative) range of TBAB.



written informed consent, sera at 258 time points from 2015 to 2018 were obtained from 162 GD patients, who were either untreated or treated with anti-thyroid drugs (ATDs) (Table 1). The TSHR autoantibody positivity for untreated GD was 96.7% by TSAbs and 90% by ECLusys TRAb when the cutoff value was set as 1.0 IU/L. Overall correlation coefficient between ECLusys TRAbs and TSAbs was 0.364 because negative correlation was observed in treated patients with hypothyroidism and GD. The sera were applied for the simultaneous measurement of TBII and TSAbs (open circles in Fig. 1B). In contrast to the distribution of TSAbs against TBII in most GD patients, that in patient M was near the *y*-axis and those in patients K and S were near the *x*-axis (Fig. 1).

We evaluated the TBII, TSAbs, and TBAb activities of stimulating type human mAbs, M22 and K1-18, and a blocking type human mAb, K1-70. TSAbs activity of the stimulating mAbs increased in a dose-dependent manner (filled circles as M22 and open circles as K1-18 in Fig. 4). In contrast, the TSAbs activity of K1-70 increased up to 125% at most, even at the maximum concentration of 200 ng/mL (open squares in Fig. 4). For comparison, we have displayed the results using mAbs superimposed on the actual data of four peculiar patients mentioned above. The relationship between TBII and TSAbs activities in patient M was similar to that of M22 or K1-18 (Fig. 4). In comparison, when IgG was purified from patient M's sera and concentrated from 1317 mg/dL to 3925 mg/dL, TBII measured by DYNOTest TRAb assay became 4.8 U/L, up from <1.0 U/L. In contrast, the relationships between TBII and TSAbs activities in patient K and S were on an extension of K1-70 (along the *x*-axis).

The addition of 80 or 160 ng/mL K1-70 to various amounts of M22 (Fig. 5A) or K1-18 (Fig. 5B) markedly decreased their TSAbs and slightly increased TBII activities (indicated by arrows). When the concentration of M22 (Fig. 5A) or K1-18 (Fig. 5B) increased from 12.5 (open circles) to 200 (filled circles) ng/mL, the approximate curves formed by K1-70 shifted to the right in a dose-dependent manner. Interestingly, these approximate curves drawn by the mixture of K1-70 and M22 or K1-18 partially but not completely paralleled with the curve formed by the sera obtained at three different time points of patient T (green squares in Fig. 5), suggesting that the sera of this patient contain some "neutral" component of TSHR-Abs. When the sera of patient M (TSAbs positive but TBII negative) and patient S (TSAbs almost negative and TBII highly positive but TBAb negative) were mixed in various combinations, another curve with a different slope was drawn (triangles in Fig. 5), which may reproduce the curve for patient M better. Moreover, the addition of sera of patient S increased TBII values having little effects on TSAbs or TBAb activities of mAbs (Fig. 6), confirming that the TSHR-Abs of patient S are mainly neutral.

When TBII values (Fig. 7A) or TSAbs activities (Fig. 7B) were plotted against the corresponding thyroid functions (*i.e.*, TSH) in the sera of GD patients shown in Table 1, there were four sera in which TBII ("a" to "d" measured with both ECLusys and DYNOTest in Fig. 7A) were disproportionately higher than TSAbs ("a" to "d" in Fig. 7B) in the euthyroid area (*i.e.*, 0.35 mU/L < TSH < 4.94 mU/L). Because these patients with GD were all in remission after the treatment with ATDs, we measured the TBAb activities in the sera of these four patients (a, b, c, and d) whether these TSHR-Abs were blocking or not. Unexpectedly, the TBAb activities in these sera were all negative (ranged from -74.0% to -4.5%), suggesting that these TSHR-Abs are mainly neutral.

### 3. Discussion

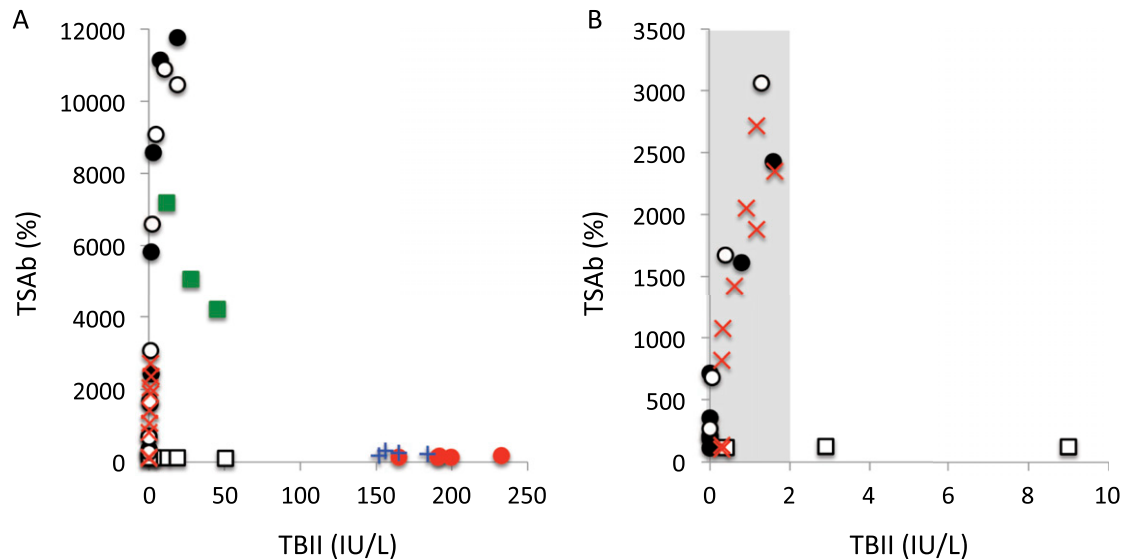
In a previous report, we described the isolation of two different TSHR mAbs, one stimulating (K1-18) and one blocking (K1-70), from the lymphocytes of a TSHR-Ab-positive patient with hypothyroidism (patient K) [3]. This proved that both stimulating and blocking TSHR-Abs can be present in a patient at the same time. In this study, we describe three additional cases whose TSHR-Abs were very suggestive. We speculated that the sera of patients K, M, and S might mainly contain blocking, stimulating, and neutral components

**Table 1. Thyroid Function and the Relationship Between TBIs (Measured by ECLusys TRAb M22) and TSAb of 162 Consecutive Patients With GD**

Patients	N	M	F	Time Points	TSH (mU/L): FT4 (ng/dL): FT3 (pg/mL):				ECLusys TRAb M22 (IU/L)				Correlation Coefficients for TRAb vs TSAb		
					0.350–4.940 <sup>a</sup>		0.70–1.48 <sup>a</sup>		1.71–3.71 <sup>a</sup>		Positive Ratio (%)			TSAb (%)	
					Mean ±SD	(Mean ±SD)	Mean ±SD	(Mean ±SD)	Mean ±SD	(Mean ±SD)	<2.0 <sup>b</sup>	<1.0 <sup>b</sup>		Mean ±SD	Positive Ratio (%): <120 <sup>b</sup>
Untreated GD	30	6	24	36	0.165 ± 0.688	2.01 ± 1.17	7.45 ± 6.66	7.58 ± 11.14	66.7	90.0	1288.9 ± 1623.7	96.7	0.535	0.0008	
Treated GD	75	12	63	122	1.326 ± 4.408	1.22 ± 0.58	4.10 ± 3.33	35.92 ± 108.84	60.7	76.2	853.0 ± 1280.1	80.3	0.522	0.0000	
GD in remission	54	7	47	84	2.220 ± 5.900	1.21 ± 0.60	3.63 ± 3.08	11.20 ± 33.90	45.9	62.4	630.9 ± 1285.5	63.5	0.650	0.0000	
Treated hypothyroid GD	3	0	3	16	2.622 ± 3.057	1.22 ± 0.15	2.66 ± 0.52	78.53 ± 90.40	66.7	66.7	1145.1 ± 2076.5	100	-0.398	0.1780	
Total	162	25	137	258	1.456 ± 4.636	1.38 ± 0.83	4.34 ± 3.99	26.87 ± 101.96	56.3	72.9	801.4 ± 1368.5	76.7	0.364	0.0000	

Abbreviations: F, female; M, male.

<sup>a</sup>Reference range.<sup>b</sup>Cutoff.

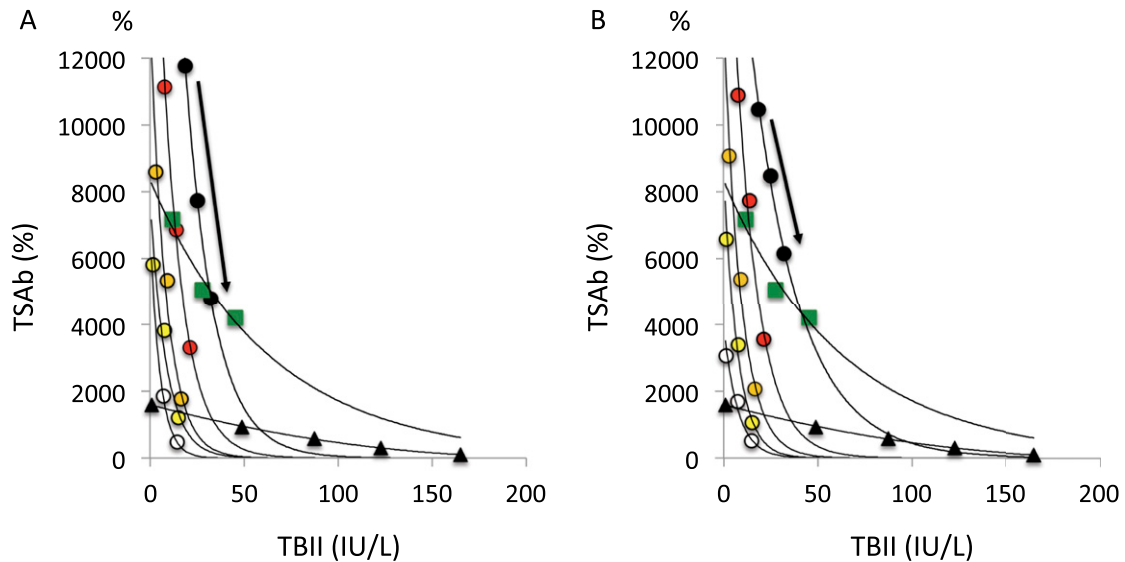


**Figure 4.** Relationship between TBII and TSAb of M22, K1-18, and K1-70 and a comparison with the four peculiar patients' sera. (A) TBII and TSAb were measured using increasing concentrations of M22 (0.4 to 200.0 ng/mL, closed circles), K1-18 (1.6 to 200.0 ng/mL, open circles), and K1-70 (10.0 to 200.0 ng/mL, open squares). TSAb values of M22, K1-18, and K1-70 were expressed against the TBII measured by DYNOTest TRAb assay based on inhibition of  $^{125}\text{I}$ -TSH binding and they were superimposed on the TSAb data of patients K (+), M (x), T (green squares), and S (red circles) shown in Fig. 1. (B) The area, in which TBII values were <10.0 IU/L and TSAb values were <3500%, was shown to compare the data of patient M and mAbs. A shadow indicates the reference (negative) range of TBII.

of TSHR-Abs, respectively. Accordingly, we assessed the TBII ability and TSAb activity of K1-18 and another stimulating M22 mAb using clinically available kits. As we expected, the TSAb activities of K1-18 and M22 were considerably high even with undetectable TBII ability. Alternatively, the TSAb distribution against TBII of K1-18 and M22 overlapped with that of patient M, suggesting that patient M's TSHR-Ab level was close to stimulating mAbs. The reason that the TSAb of stimulating mAbs are measurable in the concentration of unmeasurable TBII may be because the sensitivity of the TSAb assay is much higher than that of the TBII assay. In fact, the TBII value of patient M's serum changed to positive by its concentration. In contrast, the TSAb activity of K1-70 was kept negative even using the high doses, which show high TBII values, and the TSAb distribution against TBII of patient K was an extension of that of K1-70, suggesting that a main component of patient K's TSHR-Abs was blocking mAbs. Although, as shown in Fig. 2B, TBII values per mAb concentration of stimulating mAbs are similar to those of a blocking mAbs, the TSAb activity of stimulating mAbs can be manifested in lower concentrations (Fig. 4), but higher concentrations of blocking mAbs are necessary to exert substantial TBAb activity (Fig. 3).

Patients with GD have polyclonal TSHR-Abs that include stimulating and blocking components in their sera, and the ratio of those components may vary from patient to patient and may change during the clinical course [18]. Actually, we often experience the discrepancies that are observed in the detection of TSHR-Abs between a binding assay and bioassay in various clinical views of AITDs (Fig. 1B). Especially, patient T's TSHR-Abs changed curiously with an increase in TSAb but a decrease in TBII. Therefore, we tried to reproduce patient T's serum using different proportions of stimulating and blocking mAbs. However, patient T's curve expressed as TSAb and TBII was not paralleled very well with the lines formed by the combination of stimulating mAbs and blocking mAbs (circles in Fig. 5) but paralleled better with the line formed by the combination of the sera of patient M and patient

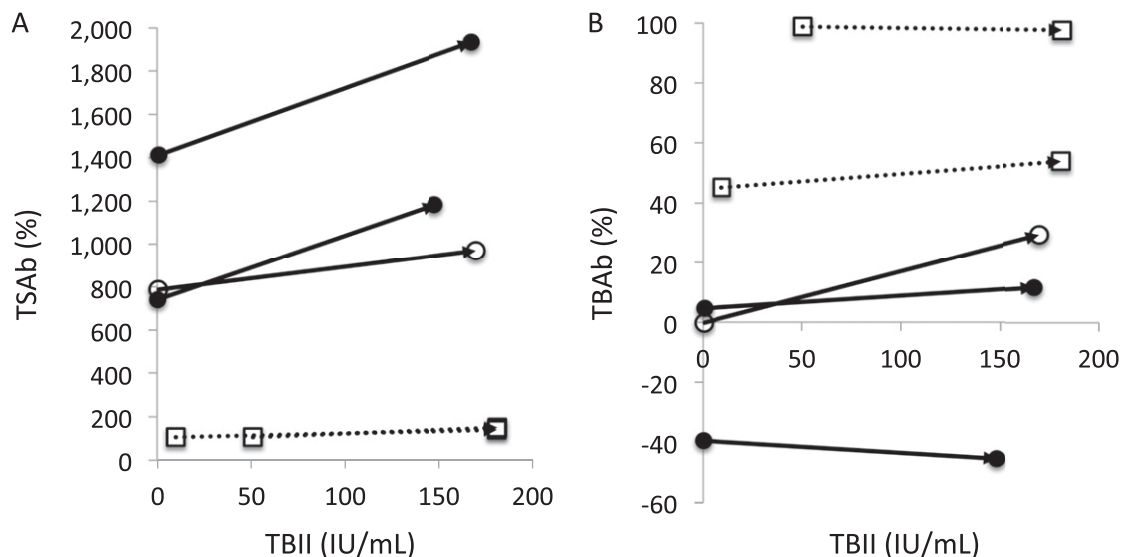




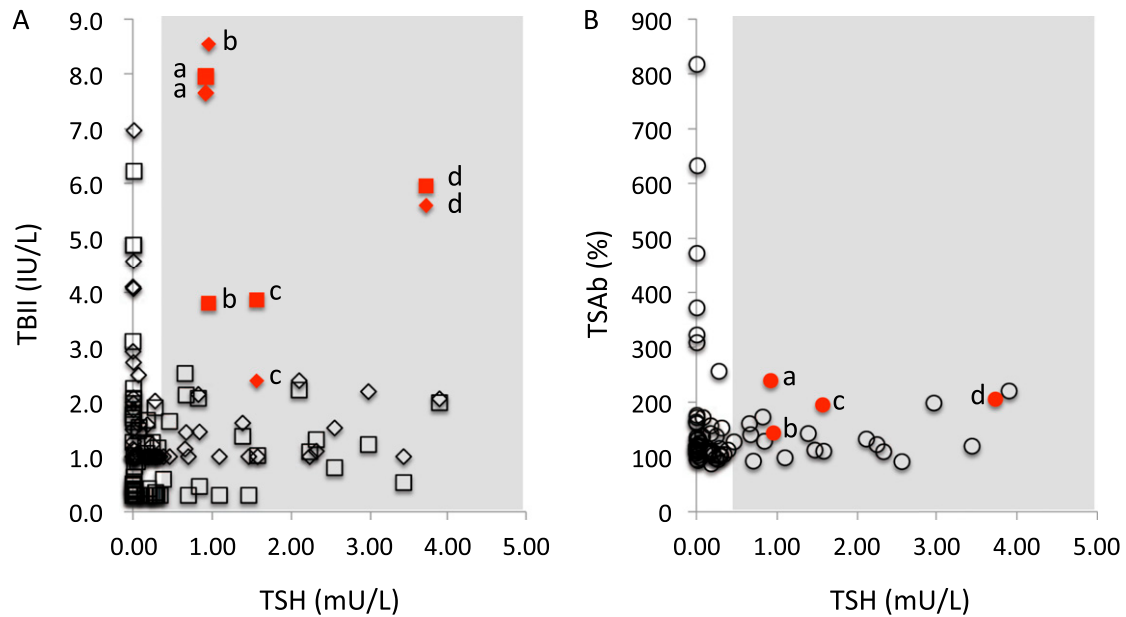
**Figure 5.** Relationship between TBII and TSAb in the mixture of M22 or K1-18 and K1-70, and a comparison with patient T. TBII and TSAb were measured using increasing concentrations of M22 (A) or K1-18 (B) (12.5 ng/mL, open; 25.0 ng/mL, yellow; 50.0 ng/mL, orange; 100.0 ng/mL, red; and 200.0 ng/mL, black circles) together with increasing concentrations of K1-70 (0, 80.0, and 100.0 ng/mL: concentrations increased along the arrow) and the TSAb were expressed against the TBII measured by DYNtest TRAb assay. Next, TBII and TSAb were measured using various combinations (4:0, 3:1, 2:2, 1:3, and 0:4) of the sera of patient M and patient S and the TSAb were expressed against the TBII measured by DYNtest TRAb assay (filled triangles). The different time points of patient T (green squares) measured by DYNtest shown in Fig. 1 were superimposed. The exponential approximate curves were drawn using Microsoft Excel<sup>®</sup>.

S (triangles in Fig. 5), suggesting that the secondary component that modified the TSAb activity in the sera of patient T was a neutral TSHR-Ab.

Orbitopathy was associated with both patient M and patient T, having high TSAb, but was not associated with patient K and patient S, who had low TSAb with a high titer of TBII.



**Figure 6.** Effect of the addition of sera, which is assumed to be neutral TSHR-Abs, on M22, K1-18, or K1-70. (A) TBII and TSAb of 4 and 8 ng/mL M22 (●), 4 ng/mL K1-18 (○), and 100 and 400 ng/mL K1-70 (□) were measured with (arrowheads) or without (normal sera) the sera of patient S and the TSAb were expressed against the TBII measured by DYNtest TRAb assay.

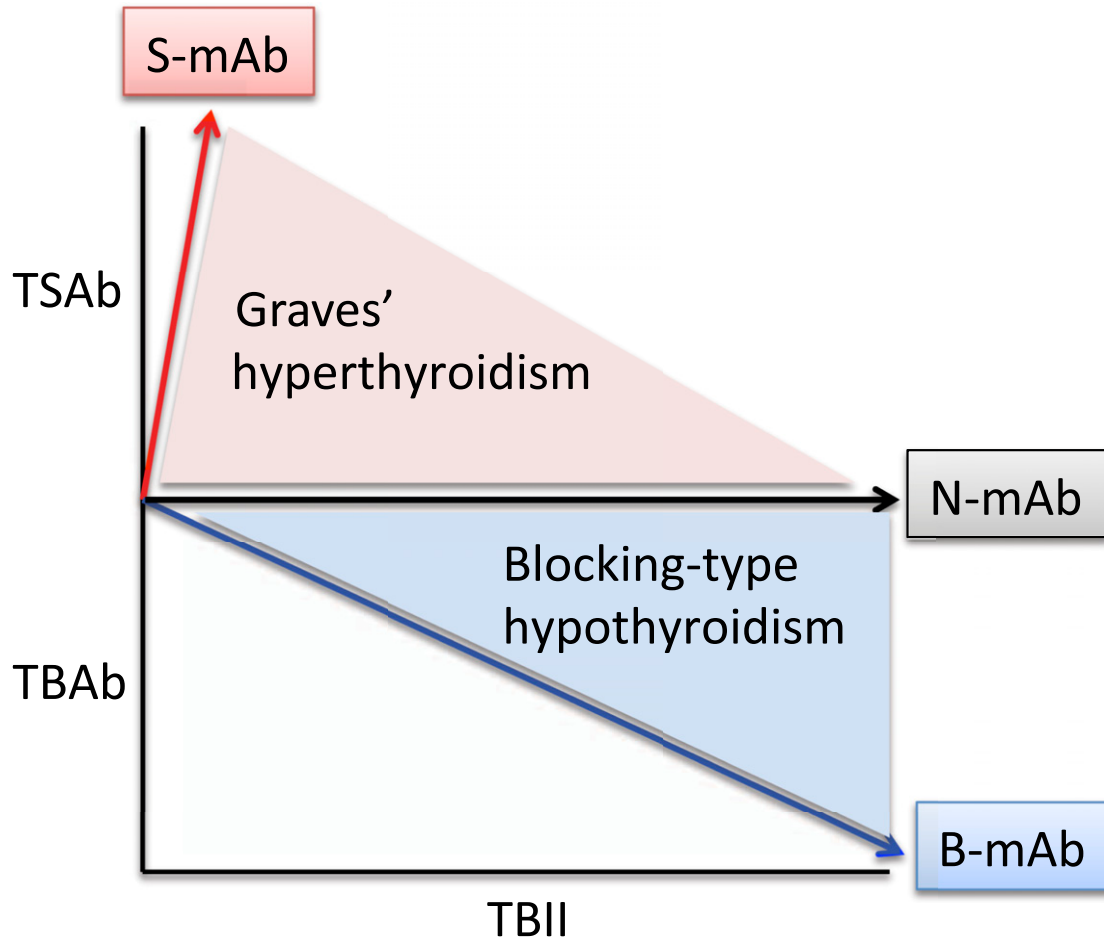


**Figure 7.** Relationship between TSH and TBII or TSAb in the patients with GD in the area of euthyroid. The TBII values (A) simultaneously measured using DYNtest TRAb assay (open diamond) or ECLusys TRAb assay (open square) and TSAb values (B, open circles) were plotted against TSH values in the sera of patients with GD shown in Table 1. The TBII of some sera (a to d) were disproportionately higher than TSAb in the euthyroid area. The TBAb activities in these four sera were all negative (a,  $-17.3\%$ , b,  $-4.5\%$ , c,  $-68.2\%$ , d,  $-74.0\%$ ). Note that these four patients are not related to patients K, M, T, or S and that there are some differences in the TBII values between DYNtest and ECLusys assays especially in “b” and “c” (interassay variation). A shadow indicates the reference range of TSH.

Similar observations of higher clinical sensitivity of the cell-based bioassay (TSAbs) using a different bioassay (Mc4) in contrast to the TBII assay in patients with GD with thyroid-associated orbitopathy have been reported. The differences between native TSHR and Mc4 are described in detail by its creators [27]. Briefly, TSHR residues 261 to 370 are replaced with LH/chorionic gonadotropin receptor in Mc4 and its properties are as follows. First, TSH-stimulated cAMP response of Mc4 was much lower than that of native TSHR, although a TSHR-Ab-stimulated cAMP response of Mc4 was similar to native TSHR. Second, blocking TSHR-Ab activity was almost absent in Mc4. Therefore, Mc4 can evaluate TSAb but not TBAb. Taken together, the TSAb component and the TBAb component may differently stimulate the orbit and the thyroid.

There are some limitations to this study. First, although the characteristics of so-called neutral TSHR-Abs that bind TSHR but neither stimulate nor inhibit TSHR are not yet established, we did not examine TSAb or TBAb activity using such neutral mAbs [6, 28]. Second, TBAb activity in the sera of patients whose TSAb levels were  $>600\%$  (data not shown) cannot be determined because of assay limitations.

Our findings can be illustrated as Fig. 8. In the Graves hyperthyroidism, various stimulating activities (TSAbs) of TSHR-Abs are consisting of the mixture of a large amount of stimulating mAbs, a small amount of blocking mAbs, and some possible neutral mAbs. The line (relationship between TSAb and TBII) composed of sera of patient M is close to the line of pure stimulating mAbs (Fig. 4B). In the blocking-type hypothyroidism, various blocking activities (TBAb) of TBII consist of the mixture of a large amount of blocking mAbs, a small amount of stimulating mAbs, and some possible neutral mAbs. Additionally, TSAb can stimulate TSHR in small amounts (Fig. 4B), but much larger amounts of TBAb are necessary to inhibit TSHR (Fig. 3B). Thus, the sera of GD may distribute the colored (pink or sky blue) area of Fig. 8.



**Figure 8.** Schematic presentation of Graves hyperthyroidism and blocking-type hypothyroidism using the relationship between TBII and TSAb or TBAbs composed of the mixture of stimulating mAbs, blocking mAbs, and possible neutral mAbs. In the Graves hyperthyroidism, various stimulating activities (TSAb) of TBII consist of the mixture of a large amount of stimulating mAbs (S-mAb), a small amount of blocking mAbs (B-mAb), and some possible neutral mAbs (N-mAb). In the blocking-type hypothyroidism, various blocking activities (TBAbs) of TBII consist of the mixture of a large amount of B-mAb, a small amount of S-mAb, and some possible N-mAb.

In conclusion, TBII activities and TSAb activities in patients with GD could be expressed as the mixture of each mAb for stimulating, blocking, and/or possible neutral TSHR-Abs.

#### 4. Recommendations and Future Directions

Measurements of TSAb may be useful in the case of TBII-negative patients with hyperthyroidism. The cloning of neutral mAbs may consolidate our conclusion.

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## Additional Information

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