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**Monoclonal autoantibodies to the TSH receptor, one with stimulating activity and one with blocking activity, obtained from the same blood sample**

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Key Words:	Thyroid

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5 **with stimulating activity and one with blocking**  
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7 **activity, obtained from the same blood sample**  
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13 Michele Evans<sup>1</sup>, Jane Sanders<sup>1</sup>, Tetsuya Tagami<sup>2</sup>, Paul Sanders<sup>1</sup>, Stuart Young<sup>1</sup>,  
14 Emma Roberts<sup>1</sup>, Jane Wilmot<sup>1</sup>, Xiaoling Hu<sup>1</sup>, Katarzyna Kabelis<sup>1</sup>, Jill Clark<sup>1</sup>, Sabrina  
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37 **Key words:** Thyroid, TSH receptor, autoantibody, Graves' disease  
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45 **Conflict of interest:** RSR Ltd is a developer of medical diagnostics including kits for  
46 measuring thyroid autoantibodies. ME, JS, PS, SY, ER, JW, XH, KK, JC, SH, TR,  
47 AC, JF and BRS are employees of RSR Ltd. TT declares no conflict of interest.  
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**ABSTRACT**

*Objective:* Patients who appear to have both stimulating and blocking TSHR autoantibodies in their sera have been described but the two activities have not been separated and analysed. We now describe the isolation and detailed characterisation of a blocking type TSHR monoclonal autoantibody and a stimulating type TSHR monoclonal autoantibody from a single sample of peripheral blood lymphocytes.

*Design, Patients and Measurements:* Two heterohybridoma cell lines secreting TSHR autoantibodies were isolated using standard techniques from the lymphocytes of a patient with hypothyroidism and high levels of TSHR autoantibodies (160 units/L by inhibition of TSH binding). The ability of the two new monoclonal antibodies (MAbs; K1-18 and K1-70) to bind to the TSHR and compete with TSH or TSHR antibody binding was analysed. Furthermore, the effects of K1-18 and K1-70 on cyclic AMP production in Chinese hamster ovary cells (CHO) cells expressing the TSHR were investigated.

*Results:* One MAb (K1-18) was a strong stimulator of cyclic AMP production in TSHR transfected CHO cells and the other (K1-70) blocked stimulation of the TSHR by TSH, K1-18, other thyroid stimulating MAbs and patient serum stimulating type TSHR autoantibodies. Both K1-18 (IgG1 kappa) and K1-70 (IgG1 lambda) bound to the TSHR with high affinity ( $0.7 \times 10^{10}$  L/mol and  $4 \times 10^{10}$  L/mol respectively) and this binding was inhibited by unlabelled K1-18 and K1-70, other thyroid stimulating MAbs and patient serum TSHR autoantibodies with stimulating or blocking activities. V-region gene analysis indicated that K1-18 and K1-70 heavy chains used the same V-region germline gene but different D and J germline genes as well as having different light chains. Consequently, the two antibodies have evolved separately from different B cell clones.

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*Conclusions:* This study provides proof that a patient can produce a mixture of blocking and stimulating TSHR autoantibodies at the same time.

For Peer Review

## INTRODUCTION

Autoantibodies to the TSH receptor (TSHR) occur in patient sera as two principal types; those with stimulating activity and those with blocking activity<sup>1-3</sup>. Stimulating TSHR autoantibodies cause hyperthyroidism whereas the much rarer blocking type TSHR autoantibodies can cause hypothyroidism<sup>1-3</sup>. Also, spontaneous transitions in the activity of serum TSHR autoantibodies do occur and this can be associated with changes in thyroid function<sup>1,4-8</sup>. There is also evidence that patient sera can contain a mixture of stimulating and blocking type TSHR autoantibodies<sup>6-8</sup>.

Recently, we have been able to prepare a thyroid stimulating monoclonal TSHR autoantibody (M22) using peripheral blood lymphocytes from a patient with hyperthyroid Graves' disease<sup>9,10</sup>. Furthermore, a similar approach has enabled the preparation of a human monoclonal TSHR autoantibody (5C9) with blocking activity from the lymphocytes of a hypothyroid patient with high levels of blocking type TSHR autoantibodies in her serum<sup>11</sup>.

We now describe the isolation of a blocking type TSHR monoclonal autoantibody and a stimulating type TSHR monoclonal autoantibody from a single peripheral blood lymphocyte preparation obtained from a patient with high levels of TSHR autoantibodies.

## MATERIALS AND METHODS

### Monoclonal antibody isolation

Lymphocytes were isolated from the peripheral blood of a 54 year old patient with hypothyroidism and high levels of TSHR autoantibodies (160 units/L) as measured by inhibition of TSH binding to TSHR coated tubes<sup>12</sup>. Local Ethical Committee approval for the study was obtained. The patient had an eight year history

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of autoimmune thyroid disease (AITD). She first presented with hyperthyroidism, was successfully treated with methimazole for 3 years but then developed hypothyroidism and was treated with thyroxine. She had been receiving thyroxine for approximately 4.5 years at the time of blood collection. The patient had a small goitre but no signs of ophthalmopathy. At the time of blood collection her serum showed strong inhibition (82% at 1/10 dilution of serum) of TSH stimulated cyclic AMP production in TSHR transfected CHO cells<sup>11</sup>. In addition, the patient's serum (1/30 dilution) on its own showed TSHR stimulating activity (313% of basal activity; >180% is positive)<sup>10</sup> (Table 1). Thyroid peroxidase antibodies were present (>500 U/mL of NIBSC reference preparation 66/387) but thyroglobulin antibody levels were undetectable (both measured in their respective direct radioimmunoassay; from RSR Ltd). IgG was purified from the patient's serum using protein A affinity chromatography on MabSelect<sup>TM</sup> (GE Healthcare, UK) as described before<sup>9-11</sup>.

The lymphocytes were infected with EBV and fused with the K6H6/B5 heterohybridoma cell line as described previously<sup>9-11</sup>. Culture supernatants were screened by assays based on inhibition of TSH binding to TSHR coated tubes<sup>12</sup> or ELISA plate wells<sup>13</sup>. Twelve thousand hybridoma clones were screened and two clones stably producing antibodies which inhibited TSH binding to the TSHR were obtained. One clone secreted an antibody designated K1-18 (IgG1 kappa) and the other clone an antibody designated K1-70 (IgG1 lambda). Both clones have been maintained in cell culture for over 24 months and are stably expressing approximately 40 mg/L of IgG.

K1-18 and K1-70 IgGs were purified from culture supernatants using protein A affinity chromatography on MabSelect<sup>TM</sup> and purity assessed by SDS-

**Deleted:** Tests for other autoantibodies (to glutamic acid decarboxylase (GAD), IA-2 and steroid 21-hydroxylase) were negative.

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polyacrylamide gel electrophoresis (PAGE). K1-18 and K1-70 IgGs were labelled with  $^{125}\text{I}$  as described in detail previously<sup>9-11</sup>.

K1-18 and K1-70 Fab fragments were prepared by treatment with mercuripapain (Sigma Aldrich Company Ltd, Poole, UK) and passed through a MabSelect column to remove any intact IgG or Fc fragments. The Fab preparations, unabsorbed by the column, were dialysed into PBS (137 mmol/L NaCl, 8.1 mmol/L  $\text{Na}_2\text{HPO}_4$ , 2.7 mmol/L KCL, 1.47 mmol/L  $\text{KH}_2\text{PO}_4$ , pH 7.4) and stored at  $-70^\circ\text{C}$ .

**Deleted:** at an IgG/enzyme ratio of 100:1 and 200:1 for 4 hours and 1 hour at  $37^\circ\text{C}$  respectively in phosphate buffered saline (PBS; 137 mmol/L NaCl, 8.1 mmol/L  $\text{Na}_2\text{HPO}_4$ , 2.7 mmol/L KCL, 1.47 mmol/L  $\text{KH}_2\text{PO}_4$ , pH 7.4 containing cysteine at a final concentration of 1 mmol/L and EDTA at a final concentration of 2 mmol/L). The reaction was stopped by addition of iodoacetamide (final concentration of 50 mmol/L) and after 30 minutes at room temperature the mixture

### Serum samples and preparations of monoclonal antibodies (MAbs) other than K1-18 and K1-70

Preparations of the human MAb M22 with powerful thyroid stimulating activity, human MAb 5C9 with strong TSH antagonist (blocking) activity and mouse MAb RSR-B2 (which blocks the stimulating activities of TSH and TSHR autoantibodies) were obtained as described before<sup>9-11,14</sup>. Also, a panel of mouse monoclonal TSHR antibodies with thyroid stimulating activity (mTSMABs) were obtained as previously described<sup>15</sup>. Negative control monoclonal antibodies used were two human MAbs to glutamic acid decarboxylase (GAD; 5B3 and 4B4)<sup>16</sup>.

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Sera containing TSHR autoantibodies with thyroid stimulating or blocking activity were obtained from patients who had given informed consent for the study. Healthy blood donor sera (HBD; Golden West Biologicals, Vista, CA) with no detectable TSHR autoantibodies were also used as controls in some experiments.

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**Deleted:** In the assay, 100  $\mu\text{L}$  of test sample (MAb preparation or patient serum) and 50  $\mu\text{L}$  of start buffer (RSR Ltd) were incubated in TSHR coated tubes for 2 hours at room temperature with gentle shaking (triplicate determination). After aspiration, the tubes were washed, 100  $\mu\text{L}$  of  $^{125}\text{I}$ -labelled protein ( $5 \times 10^4$  cpm) added and incubated for 1 hour at room temperature with shaking. The tubes were then aspirated, washed again and counted in a gamma counter.

### Inhibition of $^{125}\text{I}$ -TSH, $^{125}\text{I}$ -K1-18 or $^{125}\text{I}$ -K1-70 binding to the TSHR

Binding inhibition assays were carried out using TSHR coated tubes as described before<sup>9-12</sup>. Inhibition (%) of labelled protein binding was calculated as:-

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$$100 \times \left[ 1 - \frac{\text{cpm bound in the presence of test material}}{\text{cpm bound in the presence of control material}} \right]$$

Control material was a pool of healthy blood donor sera, individual healthy blood donor sera or other materials as indicated in the results of various experiments.

### Inhibition of M22-biotin binding to TSHR coated ELISA plates

A TSHR autoantibody ELISA based on M22 IgG labelled with biotin binding to TSHR coated ELISA wells<sup>17</sup> was used. In the assay 75  $\mu$ L of test sample was incubated with 75  $\mu$ L of start buffer (RSR Ltd) in the plate wells. After washing 100  $\mu$ L of M22-biotin was added followed by washing and incubation with 100  $\mu$ L of streptavidin peroxidase (RSR Ltd). After a final wash step, substrate was added followed by stop solution and absorbance read at 450 nm.

Inhibition (%) of M22-biotin binding was calculated as:-

$$100 \times \left[ 1 - \frac{\text{test sample absorbance at 450nm}}{\text{negative control sample absorbance at 450nm}} \right]$$

Control material was a pool of healthy blood donor sera, individual healthy blood donor sera or other materials as indicated in the results of various experiments.

### Analysis of K1-18 and K1-70 IgG binding to the TSHR

For Scatchard analysis<sup>18</sup>, unlabelled K1-18 IgG, or K1-18 Fab, or K1-70 IgG or K1-70 Fab and <sup>125</sup>I-labelled K1-18 IgG, or K1-18 Fab, or K1-70 IgG or K1-70 Fab respectively were incubated in TSHR coated tubes, aspirated, washed and counted in a gamma counter. A plot of the concentration of IgG bound against bound/free was used to calculate the affinity of binding to the TSHR.

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Deleted: in 50  $\mu$ L of assay buffer (50 mmol/L NaCl, 10 mmol/L Tris pH 7.8, 1mg/mL BSA and 1% Triton X-100) and 50  $\mu$ L of

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### Analysis of MAB biological activity in Chinese hamster ovary (CHO) cells expressing the human TSHR

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The ability of K1-18 and K1-70 IgG and other preparations to stimulate production of cyclic AMP by CHO cells expressing the human TSHR ( $5 \times 10^4$  receptors per cell) was tested in low salt buffer as described previously<sup>9,10</sup>. In addition some experiments were carried out using isotonic buffer (physiological conditions). In these experiments Krebs Ringer Hepes buffer (KRH buffer) was used (124 mmol/L NaCl, 5 mmol/L KCl, 1.25 mmol/L MgSO<sub>4</sub>, 1.45 mmol/L CaCl<sub>2</sub>, 1.25 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 25 mmol/L HEPES, 8 mmol/L glucose, 0.5 g/L bovine serum albumin, 0.5mmol/L 3 isobutyl-1-methylxanthine, pH 7.4). For analysis of the effect of MAB preparations on the constitutive activity of wild type TSHR a CHO-K1 cell line expressing approximately  $5 \times 10^5$  TSHRs per cell was used<sup>11,19</sup>.

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Deleted: In brief, cells were grown to confluence, the culture medium removed and the cells washed with 1 mL of KRH buffer. Fresh KRH buffer was then added and the cells incubated for 30 minutes at 37°C. The buffer was then removed and replaced with fresh KRH buffer containing test sample (TSH, MAB preparations, serum samples etc). The next steps were then carried out as for the experiments under the low salt buffer conditions

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The ability of K1-70 IgG and other preparations to inhibit the stimulating activity of porcine (p)TSH (RSR Ltd), recombinant human (rh) TSH (National Institute for Biological Standards and Control (NIBSC) 94/674) and native human (h) TSH (NIBSC 81/565), thyroid stimulating human MAbs M22 and K1-18, mTSMABs and patient serum TSHR autoantibodies was assessed using CHO cells expressing the TSHR<sup>11</sup>. These studies were performed by comparing the stimulatory effect of TSH, stimulating MAb or sera, in the absence or in the presence of test preparations as described previously.

### Variable region gene analysis

Heavy chain isotypes were determined using a radial diffusion assay (The Binding Site; Birmingham, UK) and light chain isotypes by Western blotting with

anti-human kappa chain and anti-human lambda chain specific mouse monoclonal antibodies (Sigma-Aldrich Company Ltd, Poole, UK).

Variable region genes of K1-18 and K1-70 heavy chains (HC) and light chains (LC) were determined as described previously<sup>9-11</sup>. Specific IgG1 HC and both kappa and lambda LC sense and antisense strand oligonucleotide primers were designed using the Medical Research Council's V-base (<http://vbase.mrc-cpe.cam.ac.uk/>) and synthesised by Sigma Genosys (Poole, UK). DNA products were cloned into pUC18 and sequenced by [GeneService \(Cambridge, UK\)](#). V region sequences were compared with available sequences of human Ig genes using Ig blast (<http://www.ncbi.nlm.nih.gov/igblast/>).

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## RESULTS

### Inhibition of <sup>125</sup>I-labelled TSH or M22-biotin binding to the TSHR,

Inhibition of <sup>125</sup>I-TSH binding to TSHR coated tubes by purified IgG isolated from the lymphocyte donor serum K1 was compared to inhibition by the 2 purified monoclonal IgGs K1-18 and K1-70 (Figure 1). The donor IgG at 100 µg/mL showed 20% inhibition, while K1-18 IgG and K1-70 IgG both showed 21% inhibition at 10 ng/mL indicating that the purified monoclonal antibodies are approximately 10,000 times more active than the donor IgG. Similar results were obtained when the unpurified donor serum (18.4 mg/mL of IgG as measured by nephelometry) was used (data not shown).

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The binding of biotin labelled M22 to TSHR coated ELISA plates was inhibited in a dose dependant manner by K1-18 IgG, K1-70 IgG and M22 IgG giving 72%, 87% and 80% inhibition respectively at 100 ng/mL and 21%, 21% and 22%

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inhibition respectively at 10 ng/mL. Results with Fab preparations were similar to those with IgG and K1-18 Fab, K1-70 Fab and M22 Fab showed 18%, 39% and 29% inhibition of TSH binding respectively at 10 ng/mL.

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### Stimulation of cyclic AMP production by MAb preparations

Figure 2A shows the abilities of a range of concentrations of K1-18 IgG, K1-18 Fab and M22 IgG to stimulate cyclic AMP production in TSHR-transfected CHO cells in low salt buffer. At 10 ng/mL K1-18 IgG gave  $25.0 \pm 1.0$  pmol/mL (mean  $\pm$  SD), K1-18 Fab  $8.3 \pm 1.9$  pmol/mL and M22 IgG  $50.3 \pm 1.6$  pmol/mL (Figure 2A). K1-70 at different concentrations up to 100  $\mu$ g/mL gave no stimulation of cyclic AMP production ( $1.2 \pm 0.3$  pmol/mL at 100  $\mu$ g/mL) compared to the buffer only control ( $1.9 \pm 0.9$  pmol/mL). The effects of K1-18 IgG, K1-18 Fab and M22 IgG were compared to the TSHR autoantibody standard NIBSC 90/672 and gave 155 units/mg, 22 units/mg and 286 units/mg respectively. The stimulating activities of K1-18 IgG, K1-18 Fab and M22 IgG were approximately 10x lower when isotonic buffer was used in the assay (data not shown). The stimulating activities of K1-18 IgG, M22 IgG and pTSH were inhibited by sera (T1 and T2) with TSH antagonist (blocking) activity (Figure 2B).

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### TSHR blocking activities of MAb preparations

K1-70 IgG inhibited the cyclic AMP stimulating activities the K1 donor serum, pTSH, rhTSH, hTSH and K1-18 IgG in a dose dependent manner with complete inhibition at 100 ng/mL K1-70 IgG (Figures 3A and B). Also K1-70 IgG, K1-70 Fab and 5C9 IgG blocked pTSH stimulation of cyclic AMP production (Figure 3C) with the dose response effects of all 3 TSHR antagonist preparations being

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2 similar. The ability of K1-70 to inhibit pTSH stimulation was approximately 3x  
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8 In the presence of K1-70 IgG (100 µg/mL final concentration), the TSHR  
9 stimulating activities of 15 different patient sera were reduced essentially completely  
10 (Figure 4). A similar degree of reduction in the stimulating activities of the same 15  
11 sera was observed with the mouse blocking MAb RSR-B2 (Figure 4). The human  
12 TSHR MAb 5C9 showed similar blocking activity to K1-70 and RSR-B2 in 14 out of  
13 the 15 sera but 5C9 had no effect on serum T11. This serum (T11) and two other sera  
14 (T5 and T8) were analysed further using different concentrations of K1-70, RSR-B2  
15 and 5C9 ranging from 0.01 µg/mL to 100 µg/mL. In the case of sera T5 and T8, all  
16 three MAbs were able to inhibit stimulation of cyclic AMP in a dose dependent  
17 manner. In contrast, with serum T11, no concentrations of 5C9 studied were effective  
18 inhibitors of stimulation whereas K1-70 and RSR-B2 showed almost complete  
19 inhibition at 0.1 µg/mL and 1 µg/mL respectively.  
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### 35 Scatchard analysis

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37 <sup>125</sup>I-labelled K1-70 IgG and Fab bound well to TSHR coated tubes and  
38 Scatchard analysis gave association constants of  $3.9 \pm 0.8 \times 10^{10}$  L/mol (mean  $\pm$ SD; n  
39 = 3) and  $2.3 \pm 0.3 \times 10^{10}$  L/mol (mean  $\pm$ SD; n = 3) respectively. Furthermore, <sup>125</sup>I-  
40 labelled K1-18 IgG and Fab bound well to TSHR coated tubes and association  
41 constants of  $6.7 \pm 1.0 \times 10^9$  L/mol (mean  $\pm$ SD; n = 3) and  $1.8 \pm 0.96 \times 10^9$  L/mol  
42 (mean  $\pm$ SD; n = 3) respectively were obtained.  
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### Inhibition of $^{125}\text{I}$ -K1-18 and $^{125}\text{I}$ -K1-70 IgG binding to the TSHR by patient serum TSHR autoantibodies and other TSHR antibodies

Binding of  $^{125}\text{I}$ -K1-18 or  $^{125}\text{I}$ -K1-70 IgG to TSHR coated tubes was not markedly influenced by sera from 10 different healthy blood donors (0.0 – 14.7% inhibition) and (0.0 – 5.6% inhibition) respectively. Sera from 20 patients with Graves' disease, all positive for TSHR autoantibodies as assessed by  $^{125}\text{I}$ -TSH binding inhibition assays<sup>12</sup>, inhibited  $^{125}\text{I}$ -K1-18 and  $^{125}\text{I}$ -K1-70 binding to TSHR coated tubes (inhibition range 23.2 – 79.5% and 19.2 – 77.6% respectively). Inhibition of  $^{125}\text{I}$ -K1-18 and  $^{125}\text{I}$ -K1-70 binding to TSHR coated tubes correlated well with inhibition of  $^{125}\text{I}$ -TSH binding to TSHR coated tubes ( $r = 0.94$  and  $0.93$  respectively) and  $^{125}\text{I}$ -K1-18 and  $^{125}\text{I}$ -K1-70 binding to TSHR coated tubes also correlated well with each other ( $r = 0.87$ ). Labelled K1-18 and K1-70 binding to the TSHR was inhibited in a dose dependent manner by the donor serum K1, and patient serum TSHR autoantibodies with either stimulating or blocking (TSH antagonist) activities and gave a similar dose response to labelled TSH inhibition by the same sera (data not shown). Labelled K1-18 and K1-70 IgG binding to the TSHR was also inhibited in a dose dependant manner by M22, 5C9, RSR-B2 and mTSMABs 1-7<sup>15</sup> (data not shown).

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### Effects of MAbs on TSHR constitutive activity

K1-70 IgG or RSR B2 IgG had no effect on TSHR wild type constitutive activity, whereas 5C9 IgG inhibited constitutive TSHR activity by 61% at 1  $\mu\text{g}/\text{mL}$ .

**Deleted:** The effects of K1-70, 5C9 and RSR-B2 on TSHR wild type constitutive activity are shown in Table 3.

**Deleted:** Neither K1-70 nor RSR-B2 IgG influenced constitutive activity

### Variable region sequences of K1-18 and K1-70

K1-18 IgG has an IgG1 HC and kappa LC. Sequence analysis of the genes coding for K1-18 IgG showed that the HC V region was from the VH5-51\*01

germline gene, the D region from the D3-16\*02 gene and the J region from the JH3\*02 or \*01 gene. The K1-18 LC V region was derived from the V3-20\*01 germline gene and the J region from the JK-1\*01 gene. The K1-18 HC CDR1 is 5 amino acids long, its CDR2 is 17 amino acids long and CDR3 is 13 amino acids long while the K1-18 LC CDR1 is made up of 12 amino acids, its CDR2 of 7 amino acids and CDR3 of 9 amino acids. There are somatic mutations and insertions in both the K1-18 HC and LC gene sequences compared to the germline gene sequences (Table 2).

K1-70 IgG has an IgG1 HC and lambda LC. Sequence analysis of the genes coding for K1-70 IgG showed that the HC V region was from the VH5-51\*01 germline gene, the D region from the D1-7\*01 gene and the J region from the JH4\*02 gene while the K1-70 LC V region was derived from the VL1-51\*01 germline gene combined with a J region from the LJ7\*1 gene (Table 2). The K1-70 HC CDR1 is 5 amino acids long, its CDR2 is 17 amino acids long and CDR3 is 10 amino acids long while the K1-70 LC CDR1 is made up of 13 amino acids, its CDR2 of 7 amino acids and CDR3 of 11 amino acids. There are somatic mutations and insertions in both the K1-70 HC and LC gene sequences compared to the germline gene sequences (Table 2).

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Variable region sequences of K1-70¶

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## DISCUSSION

There are several reports which suggest that TSHR autoantibodies with stimulating activity and with blocking activity can be present in the same serum sample<sup>6-8</sup>. Isolation of K1-18 and K1-70 from the same preparation of lymphocytes now provides proof of this concept. The blocking type MAb K1-70 has a similar high affinity for the TSHR ( $4 \times 10^{10}$  L/mol) as the blocking type human MAb (5C9) which we isolated recently from a different patient using the same procedure<sup>11</sup>, and is in the



range ( $3-7 \times 10^{10}$  L/mol) observed for patient serum blocking type TSHR autoantibodies<sup>20</sup>. The stimulating MAb K1-18 is also high affinity ( $0.7 \times 10^{10}$  L/mol) although this is somewhat lower than that of the thyroid stimulating human MAb M22 ( $5 \times 10^{10}$  L/mol)<sup>9,10</sup>.

To date only four human TSHR MAbs have been isolated which have the characteristics of patient serum TSHR autoantibodies and all four are IgG1 subclass. However kappa or lambda light chains are found in human monoclonal TSHR autoantibodies whether they have stimulating or blocking activity.

Both the K1-18 and the K1-70 V region genes underwent antigen driven maturation by somatic mutation and insertion in both the HC and LC genes (Table 2). It is of interest that the same HC V region germline gene VH5-51\*01 (VH5 family) was used by both K1-18 and K1-70 however as these are combined with different D and J genes, the two antibodies have evolved separately from each other and must have originated from different B cell clones. The thyroid stimulating human monoclonal autoantibody M22 HC V region genes are derived from a germline (VH32) which also belongs to the VH5 gene family<sup>9,10</sup> suggesting that this small family of genes may have a significant role in the formation of some TSHR autoantibodies (with either thyroid stimulating or blocking activity). However, the HC V region genes of the human blocking monoclonal autoantibody (5C9) which acts as an inverse agonist are derived from the VH3 family (VH3-53)<sup>11</sup>.

K1-18 IgG was less active than M22 IgG in terms of stimulating cyclic AMP production in TSHR transfected CHO cells (Figure 2A-) although similar maximum responses were obtained with both antibodies. This lower activity of K1-18 is consistent with its lower affinity for the TSHR. K1-18 Fab stimulating activity was considerably lower than that of the intact IgG (Figure 2A) and this was also consistent

**Deleted:** The stimulating MAb K1-18 is IgG1 subclass with a kappa light chain (M22 stimulating MAb is IgG1/lambda) and the blocking MAb K1-70 is IgG1 with a lambda light chain (5C9 blocking MAb is IgG1/kappa). Analysis of RNA from the K1-18 and K1-70 clones confirmed that the antibodies were monoclonal.

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with the lower affinity of the Fab preparation. In contrast M22 Fab has similar affinity for the TSHR to intact IgG and M22 Fab is a more active stimulator than the intact IgG<sup>10,20</sup>. The molecular basis for this difference between M22 and K1-18 is not clear at present but maybe due to differences in their binding kinetics to the TSHR. For example dissociation of labelled M22 IgG bound to the TSHR did not occur following the addition of unlabelled M22 IgG whereas 24% dissociation of labelled K1-18 IgG bound to the TSHR was observed within 3 hours after adding unlabelled M22 IgG<sup>20</sup> (data not shown). The TSHR stimulating activity of K1-18 was blocked by patient sera containing blocking type TSHR autoantibodies (Figure 2B) and by K1-70 (Figure 3A). Also, the TSHR stimulating activity in 15/15 Graves' sera were blocked by K1-70 (Figure 4). Consequently K1-70 has the characteristics of patient serum TSHR autoantibodies with blocking activity and K1-18 the characteristics of patient serum TSHR autoantibodies with stimulating activity.

A mixture of K1-18 and K1-70 (Table 1 and Figure 3A) or a mixture of TSH, K1-70 and K1-18 (data not shown) in the appropriate proportions has the weak thyroid stimulating activity characteristic of the donor serum.

K1-70 inhibited the stimulating activity of all TSHR agonists tested (pTSH, hTSH, M22, K1-18 and patient sera with thyroid stimulating activity and the stimulating activity of the K1-donor serum). The observations that K1-70 is able to inhibit both the stimulating activity of the K1-donor serum (Figure 3B) and K1-18 (Figure 3A), which was isolated from the same patient emphasises that the interaction between blocking and stimulating antibodies in the patient is complex. The clinical symptoms observed in autoimmune thyroid disease are dependent in part at least on the relative concentrations and activities of blocking and stimulating autoantibodies in the serum at any one time (ie the sum of the activities of the

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1 autoantibodies present in the sera)<sup>1,4-6</sup>. Their combined effects on TSH interactions  
 2 with the TSHR may also be a factor. Furthermore, the concentrations and/or activities  
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 6 of stimulating or blocking TRAb may vary in the same patient during the course of  
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 8 the disease and indeed fluctuation of symptoms from hyperthyroidism to  
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 10 hypothyroidism in the same patient over time are well known<sup>1,4-8</sup> and was observed  
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 12 with the lymphocyte donor of this study.

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 17 stimulating activity which can also act as an antagonist of stronger stimulators such as  
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 19 TSH<sup>21</sup> has lead to speculation that TSHR autoantibodies of this type may be present in  
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 21 patients with autoimmune thyroid disease<sup>22</sup>. So far however, human TSHR MAbs  
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 23 with weak agonist activity combined with an ability to act as antagonists of TSH have  
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 25 not been isolated. In particular, K1-18 and M22 are pure TSHR agonists and K1-70  
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 27 and 5C9 are pure TSHR antagonists<sup>2,9-11</sup>.

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 29 Studies on the binding of <sup>125</sup>I-labelled K1-18 or K1-70 to TSHR coated tubes  
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 31 indicated that the binding of both monoclonal antibodies was inhibited by unlabelled  
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 33 preparations of each other, M22, 5C9 and patient serum TSHR autoantibodies with  
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 35 stimulating or with blocking activity. Furthermore, both K1-18 and K1-70  
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 37 (unlabelled) inhibit the binding of labelled TSH or M22 to the TSHR. Consequently,  
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 39 the epitopes on the TSH receptor which interact with K1-18 and with K1-70 are likely  
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 41 to be closely related to the regions on the TSHR which interact with all these other  
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 45 K1-70 differs from the 5C9, the other human TSHR MAb with blocking  
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 47 activity we have isolated<sup>11</sup>, in terms of its effect on TSHR constitutive activity. In  
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 49 particular, 5C9 has a marked inhibiting effect on constitutive cyclic AMP production  
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 51 in TSHR transfected CHO cells<sup>11</sup> whereas K1-70 has no effect. Further differences

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between the two monoclonal antibodies were also observed in 1/15 Graves' sera (Figure 4). The TSHR stimulating activity in this one serum sample could not be blocked by 5C9 but was completely blocked by K1-70. This suggests that although stimulating autoantibodies in different patient sera interact with the same region of the TSHR there are differences in the actual contact amino acids involved. Furthermore, this observation indicates that there are subtle differences in the epitopes recognised by 5C9 and K1-70 even though K1-70 inhibits binding of 5C9 or TSH to the TSHR effectively<sup>11</sup>. Such subtle differences in the interaction of K1-70 and 5C9 with the receptor may be responsible for their different effects on the constitutive activity.

Deleted: 5 and Table 2

K1-18 and K1-70 IgGs were in the region of 10,000x more potent than the K1 donor serum in inhibition of <sup>125</sup>I-TSH binding assays. Similar ratios between the activity of the monoclonal TSHR autoantibody IgG and donor serum IgG were observed in the case of M22 (approximately 3,000x)<sup>9,10</sup> and 5C9 (approximately 4,000x)<sup>11</sup>. This particular property of K1-18, K1-70, M22 and 5C9 distinguishes them clearly from human monoclonal autoantibodies reactive with the TSHR described in earlier reports<sup>23-26</sup>. In particular, the monoclonal antibodies described earlier were of similar potency to the donor serum IgG<sup>23-25</sup> and those with TSHR stimulating activity did not have the ability to inhibit TSH binding to the TSHR<sup>27,28</sup>.

In summary therefore, K1-70 is a powerful antagonist of both TSH and thyroid stimulating antibodies while K1-18 is a strong stimulator of cyclic AMP production in CHO cells expressing the TSHR, both K1-70 and K1-18 are characterised by high affinity binding to the TSHR. Both K1-70 and K1-18 binding to the TSHR is inhibited by patient serum autoantibodies with either stimulating or blocking activity as well as by thyroid stimulating or blocking MAbs.

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The ability of K1-70 to act as a powerful antagonist of thyroid stimulating autoantibodies (and TSH) suggests potentially important *in vivo* applications. In particular, K1-70 preparations (or molecules derived from them) could be used as specific inhibitors of thyroid stimulation by TSHR autoantibodies in patients with Graves' disease. In addition K1-70 may be helpful in modulating the clinical activity of Graves' ophthalmopathy by targeting the TSHRs expressed in orbital adipocytes and blocking the stimulating effect of TSH and thyroid stimulating autoantibodies on IL-6 expression in orbital preadipocyte fibroblasts and IL-6 secretion by mature adipocytes<sup>29,30</sup>. The stimulating activity of K1-18 suggests that this human monoclonal antibody has potential as an alternative to recombinant TSH in various *in vivo* applications<sup>31,32</sup> as proposed for other thyroid stimulating monoclonal antibodies including M22<sup>2,29</sup>. Also, the availability of two human monoclonal autoantibodies to the TSHR with distinct biological activities (one stimulating and one blocking) from the same patient together with M22 and 5C9 provides new opportunities for analysing the relationship between binding and biological activity of blocking and stimulating autoantibodies without the bias associated with the use of polyclonal patient serum preparations.

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For Peer Review



**Table 1** Effect of donor serum on cyclic AMP activity in CHO cells expressing the TSH receptor

Dilution of K1 donor serum <sup>1</sup>	Stimulation of cyclic AMP production (%; >180% is positive) <sup>2</sup>
1/10	280
1/30	313
1/100	220
1/300	130
Dilution of K1 donor serum <sup>1</sup>	Inhibition of TSH mediated cyclic AMP stimulation (%; >30% is positive) <sup>3</sup>
1/10	82
1/20	29
1/40	9
1/80	0

<sup>1</sup>Final dilution in cyclic AMP buffer (NaCl free Hank's buffered salts solution containing 1 g/L glucose, 20 mmol/L Hepes, 222 mmol/L sucrose, 15 g/L bovine serum albumin and 0.5 mmol/L 3-isobutyl-1-methyl xanthine pH 7.4).

<sup>2</sup>100% = cyclic AMP concentration in the presence of a pool of healthy blood donor sera diluted 1:10 in cyclic AMP assay buffer

<sup>3</sup>0% = cyclic AMP concentration in the presence of 3 ng/mL porcine TSH and a pool of healthy blood donor sera diluted 1:10 in cyclic AMP assay buffer.

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**Table 2** Germline genes used by four human monoclonal TSHR autoantibodies

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Antibody Chain	Germline	DNA % homology with germline	V(D)J R/S mutations ratio in framework and CDRs	No of mutations in framework (replacement)	No of mutations in CDRs (replacement)
K1-18 HC (IgG 1)	VH5-51*01 D3-16*2 JH3*02	97% 87% 100%	2.7 (+8bp insertion V/D)	4(3)	7(5)
K1-18 LC ( $\kappa$ )	V3-20*01 JH1*01	98% 97%	5.0 (+3bp insertion V/J)	1(0)	5(5)
K1-70 HC (IgG 1)	VH5-51*01 D1-7*01 JH4*02	96% 100% 97%	6.0 (+5bp insertion V/D + 12bp insertion D/J)	9 (9)	5(3)
K1-70 LC ( $\lambda$ )	VL1-51*01 LJ7*01	97% 100%	2.0 (+ 2bp insertion V/J)	4(2)	5(4)

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## FIGURE LEGENDS

Figure 1 Effect of human monoclonal antibodies and donor serum IgG on  $^{125}\text{I}$ -TSH binding to TSHR coated tubes. Results shown are means  $\pm$ SD (n = 3). 4B4 is a human monoclonal antibody to glutamic acid decarboxylase (negative control); HBD = healthy blood donor.

- A K1-18 IgG (▲ solid line)  
K1-70 IgG (● solid line)  
4B4 IgG (■ solid line)  
4B4 Fab (■ dashed line)
- B Donor serum IgG (◇ solid line)  
HBD IgG (× solid line)

Figure 2 Effect of human monoclonal antibodies on (A) stimulation of cyclic AMP production in TSHR transfected CHO cells and (B) effect of patient sera with TSHR blocking activity (T1 and T2) on various thyroid stimulators. Results shown are means  $\pm$ SD (n = 3). 5B3 and 2G4 are human monoclonal antibodies to GAD and to TPO respectively (negative controls).

- A K1-18 IgG (▲ solid line)  
K1-18 Fab (▲ dashed line)  
M22 IgG (◆ solid line)  
2G4 IgG (× solid line)

- B porcine TSH 3 ng/mL (□)  
K1-18 IgG 10 ng/mL (■)  
M22 IgG 3 ng/mL (■)

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T1 alone diluted 1:10 (final concentration) in cyclic AMP assay buffer (□)

T2 alone diluted 1:10 (final concentration) in cyclic AMP assay buffer (□)

Figure 3

**A** Blocking effect of K1-70 MAb on cyclic AMP stimulation by K1-18 IgG, porcine TSH, human TSH and recombinant human TSH

K1-18 IgG 10 ng/mL (▲ solid line)

porcine TSH 3 ng/mL (× solid line)

human TSH 100 ng/mL (+ solid line)

recombinant human TSH 100 ng/mL (○ solid line)

**B** Blocking effect of K1-70 MAb (◆ solid line) on donor serum stimulating activity. Cyclic AMP concentration with buffer only = 2.7 ±0.3 pmol/mL. K1-donor serum was used at a final dilution of 1:30

**C** Blocking effect of K1-70 IgG, K1-70 Fab and 5C9 IgG on cyclic AMP stimulation by porcine TSH (3 ng/mL).

K1-70 IgG (● solid line)

K1-70 Fab (● dashed line)

5C9 IgG (□ solid line)

Results shown are means ±SD (n = 3)

**Deleted:** Figure 3 . Effect of patient sera with TSHR blocking activity (T1 and T2) on various thyroid stimulators. Results shown are means ±SD (n = 3).  
 porcine TSH 3 ng/mL (□)  
 K1-18 IgG 10 ng/mL (■)  
 M22 IgG 3 ng/mL ( )  
 T1 alone diluted 1:10 (final concentration) in cyclic AMP assay buffer ( )  
 T2 alone diluted 1:10 (final concentration) in cyclic AMP assay buffer ( )

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Figure 4 Effect of blocking MAbs K1-70, 5C9 and RSR-B2 on the stimulating activities of 15 different Graves' sera. Results shown are means  $\pm$ SD (n = 3); 5B3 is a monoclonal antibody to GAD (negative control).

+5B3 at final concentration 100  $\mu$ g/mL (□ )

+K1-70 at final concentration 100  $\mu$ g/mL (□)

+5C9 at final concentration 100  $\mu$ g/mL (■)

+RSR-B2 at final concentration 100  $\mu$ g/mL ( )■

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 . . . porcine TSH 3 ng/mL (× solid line)¶  
 . . . human TSH 100 ng/mL (+ solid line)¶  
 . . . recombinant human TSH 100 ng/mL (O solid line)

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 5C9 IgG (□ solid line)

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**Deleted:** Figure 6 . Effect of various patient sera on binding of  $^{125}$ I-labelled (A) K1-18, (B) K1-70 and (C) TSH to TSHR coated tubes.¶  
 K1 donor serum (× solid line)¶  
 . . Blocking serum B1 (□ solid line)¶  
 . . Blocking serum B2 (■ solid line)¶  
 . . Stimulating serum S1 (O solid line)¶  
 . . Stimulating serum S2 (● solid line)¶

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Figure 1A

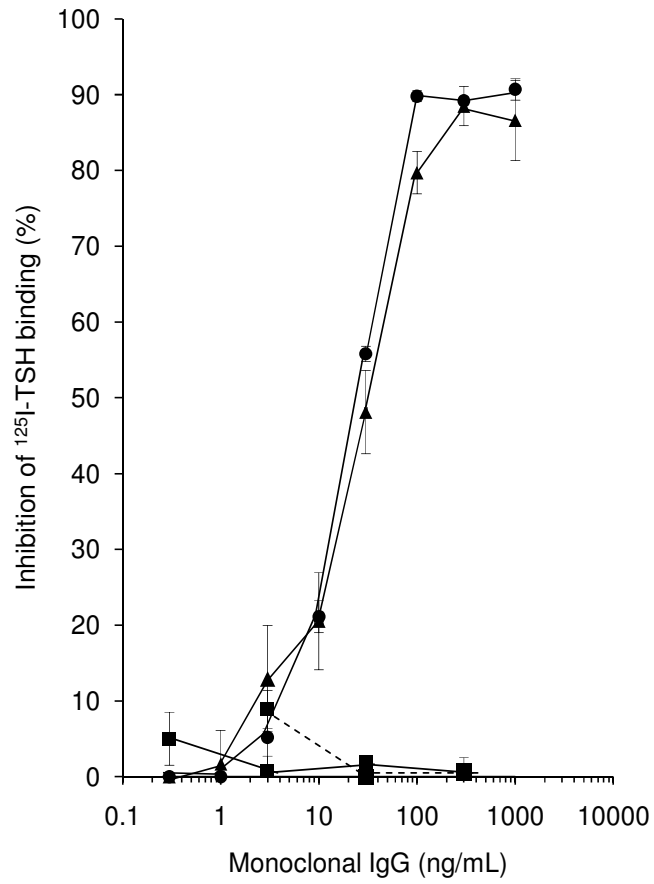
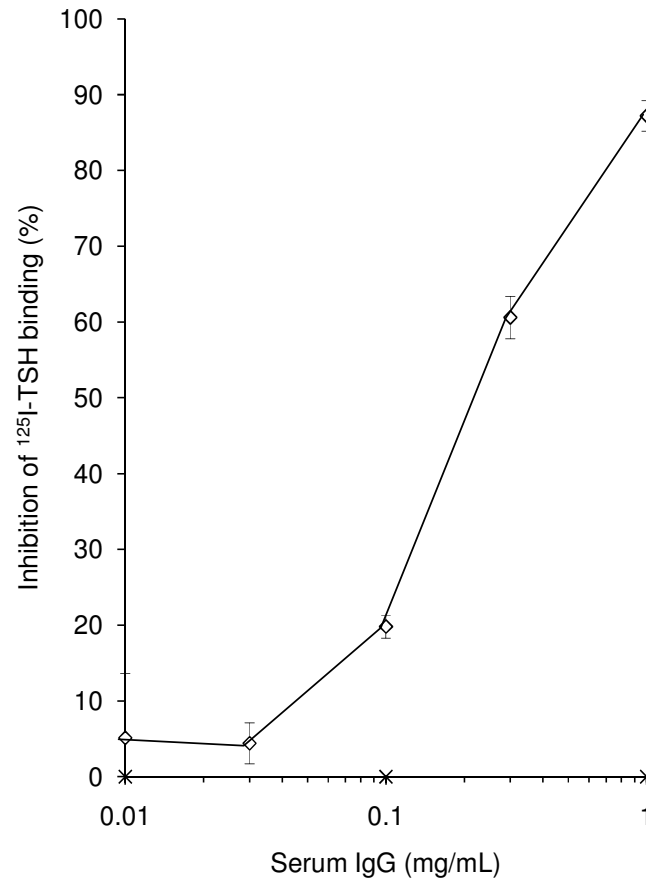


Figure 1B

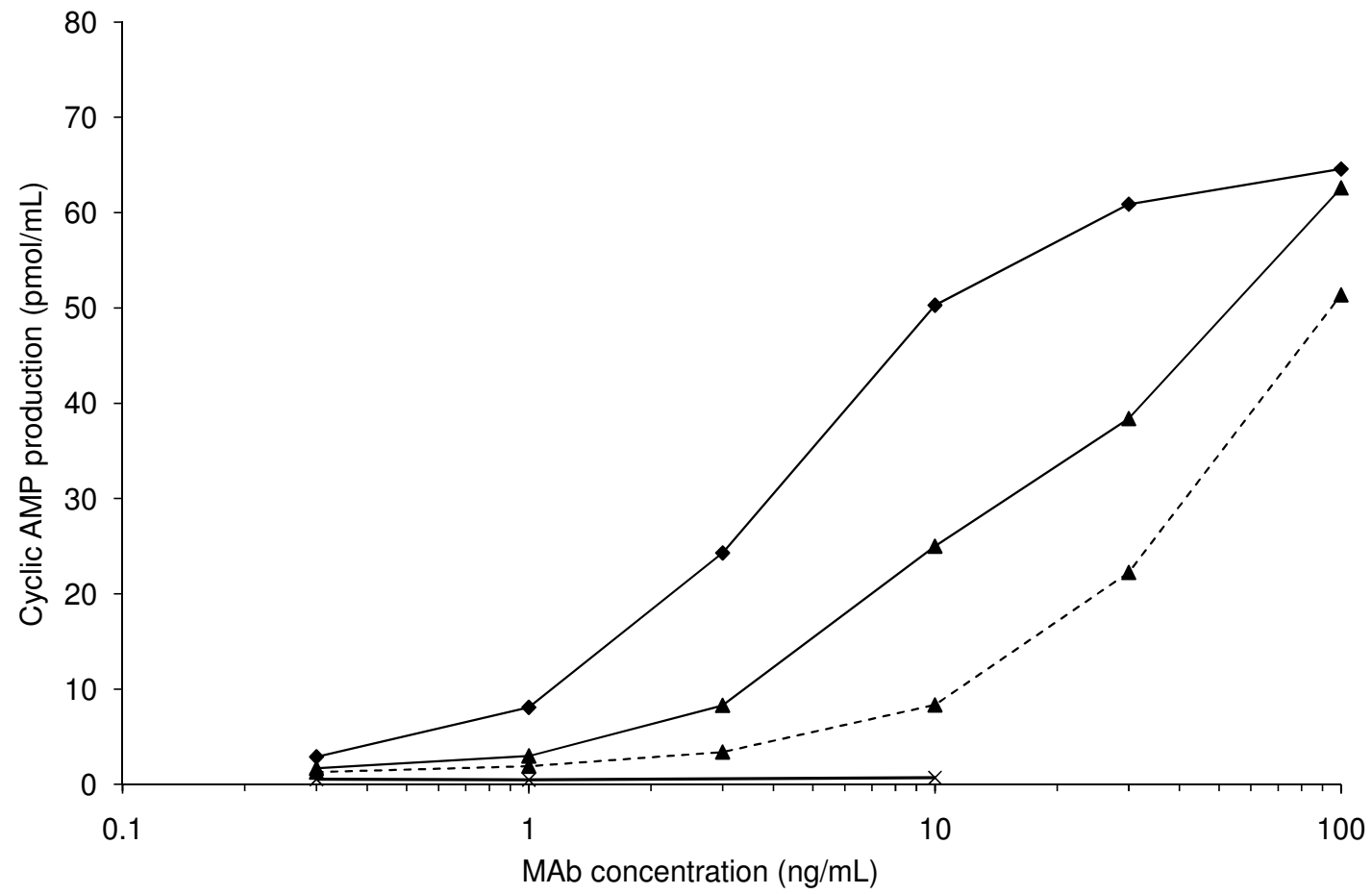


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Figure 2A

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Figure 2B

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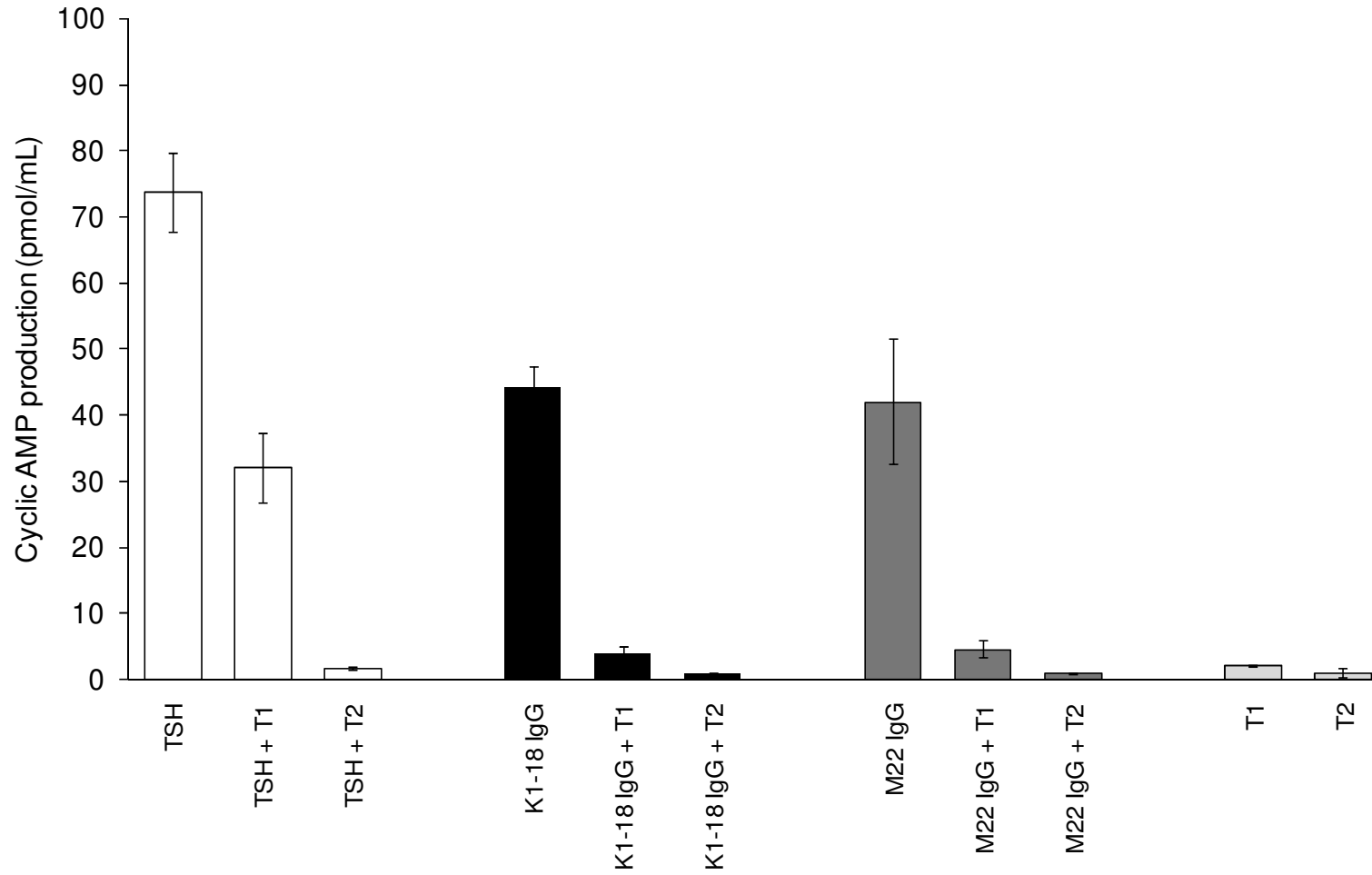
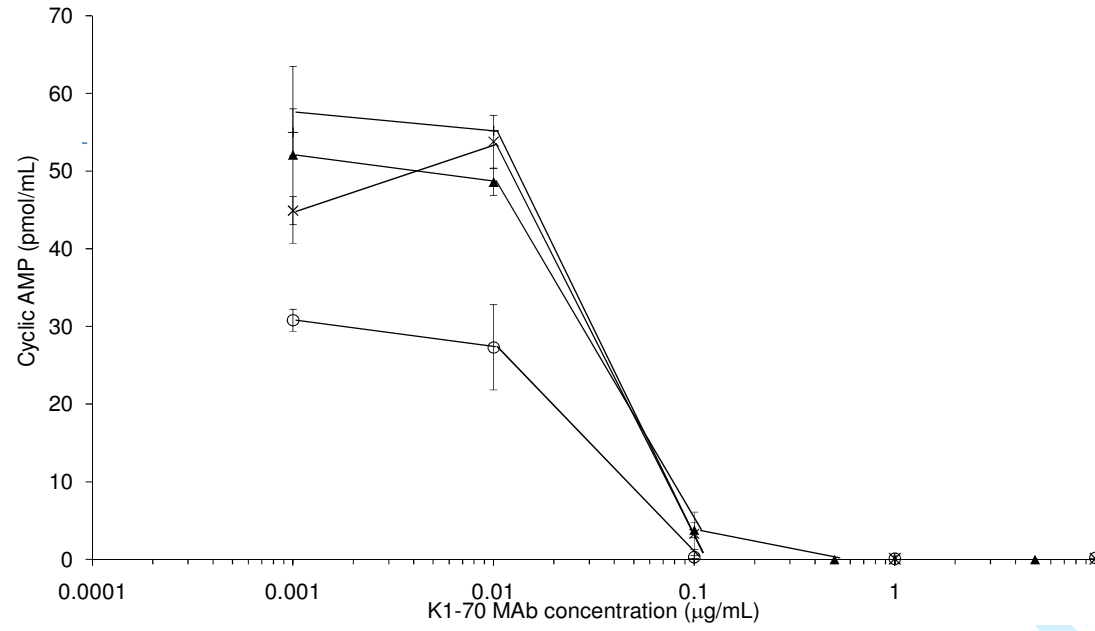




Figure 3A

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Figure 3B

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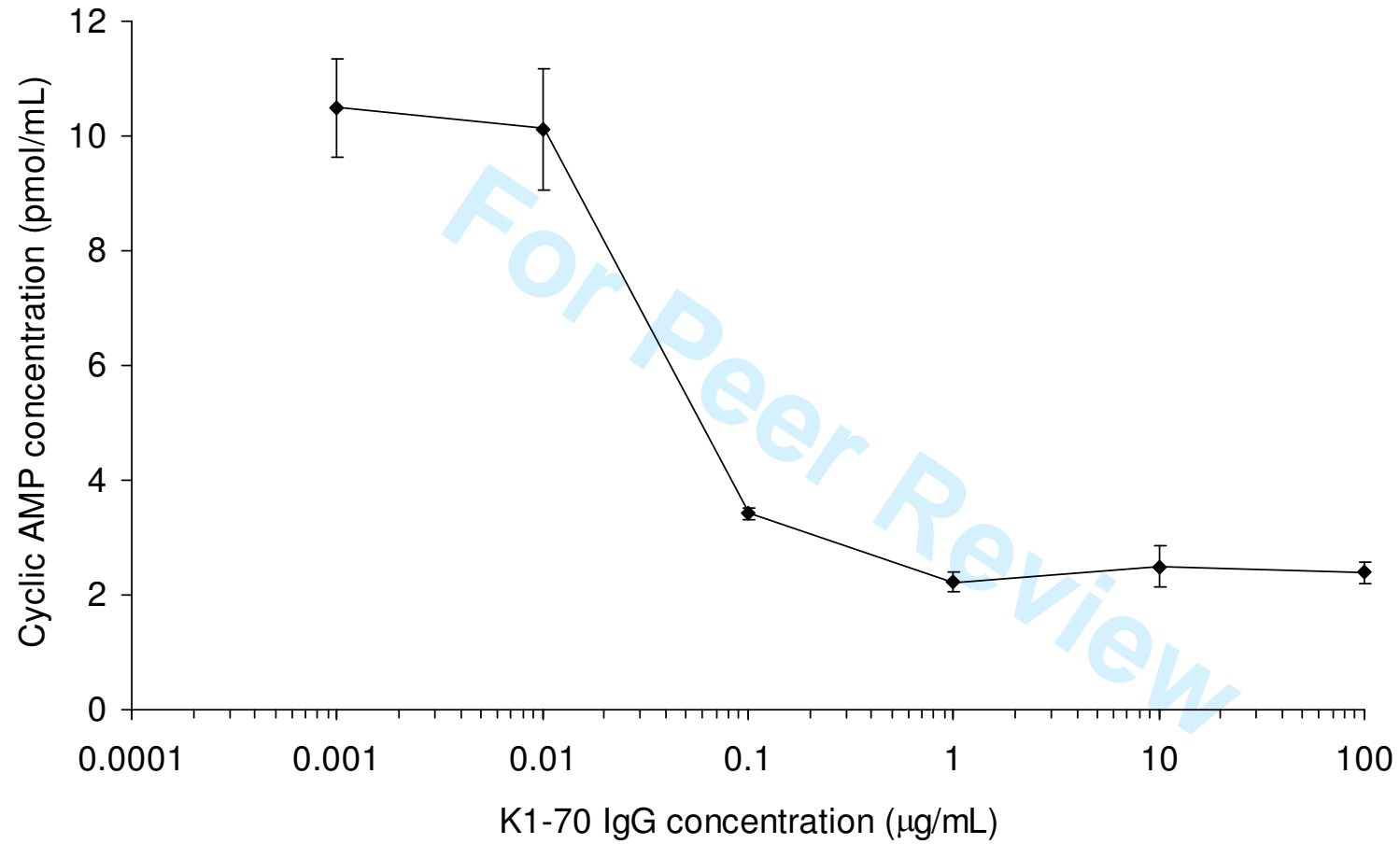
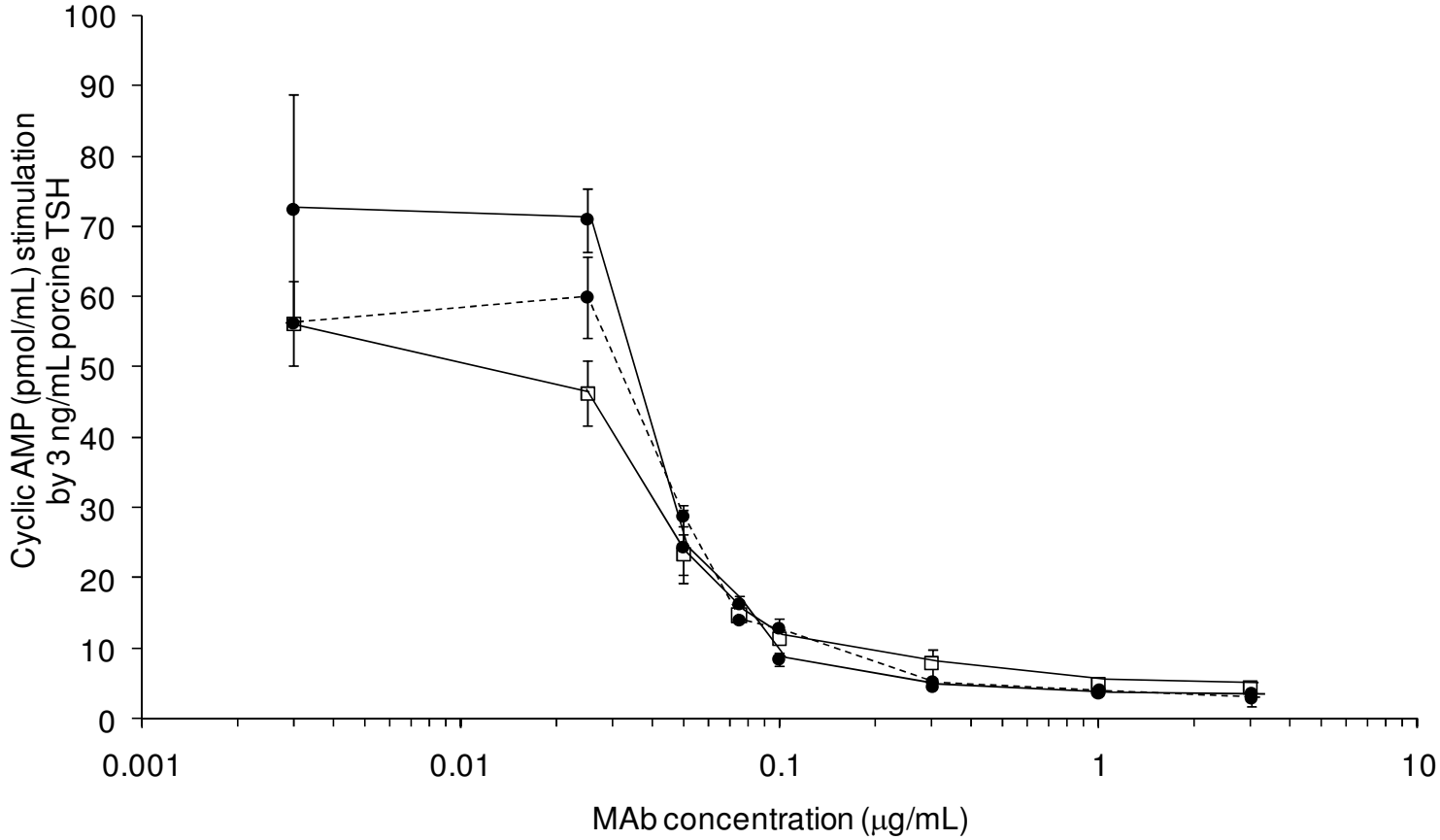


Figure 3C

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Figure 4

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