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Graves’ Disease TSHR-Stimulating Antibodies (TSAbs) Induce the Activation of Immature Thymocytes: A Clue to the Riddle of TSAds Generation?

Mireia Giménez-Barcons,* Roger Colobran,*† Ana Gómez-Pau,* Ana Marín-Sánchez,‡ Anna Casteràs,§ Gabriel Obiols,§ Raúl Abella,§ Joaquín Fernández-Doblas,§ Massimo Tonacchera,‖ Ana Lucas-Martín,‡,# and Ricardo Pujol-Borrell*†‡

Graves’ disease (GD) is an autoimmune thyroid disease defined by the production of stimulating autoantibodies to the thyroid-stimulating hormone receptor (TSHR) (TSAds) that induce a sustained state of hyperthyroidism in patients. We previously demonstrated that TSHR, the target of this autoimmune response, is also a key susceptibility gene for GD, probably acting through thymic-dependent central tolerance. We also showed that TSHR is unexpectedly, expressed in thymocytes. In this report, we confirm the expression of TSHR in thymocytes by protein immunoblotting and quantitative PCR, and show that expression is confined to maturing thymocytes. Using functional assays, we show that thymic TSHR is functional and that TSAds can stimulate thymocytes through this receptor. This new activity of TSAds on thymocytes may: 1) explain GD-associated thymic enlargement (hyperplasia), and 2) suggest the provocative hypothesis that the continuous stimulation of thymocytes by TSAds could lead to a vicious cycle of iterative improvement of the affinity and stimulating capability of initially low-affinity antibacterial (e.g., Yersinia) Abs cross-reactive with TSHR, eventually leading to TSAds. This may help to fill one of the gaps in our present understanding of unusual characteristics of TSAds. The Journal of Immunology, 2015, 194: 4199–4206.

Graves’ disease (GD) is a clinical form of autoimmune thyroid disease (AITD) defined by the production of stimulating autoantibodies to the thyroid-stimulating hormone (TSH) receptor (TSHR) (TSAds) (1, 2) that induce a sustained state of hyperthyroidism. In most GD cases there is also production of autoantibodies to thyroid peroxidase and/or thyroglobulin (Tg), thus indicating the concomitant failure of tolerance to several thyroid Ags (3, 4). Immunohistopathological changes occurring in the thyroid gland of GD patients, such as inappropriate HLA expression (5) and the formation of tertiary lymphoid tissue (6), are similar to those found in chronic infections (7), suggesting that the intrathyroidal immune response is important for disease pathogenesis (8). Genome-wide transcriptomic analysis of GD thyroid showed a strong IFN signature (9, 10) and a pattern of immune system–related genes of progressive complexity over time, supporting the importance of thyroid-centered mechanisms in chronification (11).

The TSHR, thyroid peroxidase, and Tg–reactive autoantibodies in GD have the features of T cell–dependent Ab responses, which require the help of specific T cells. For these T cells to be available, they have to escape deletion in the thymus and, therefore, a failure of central tolerance is required (12).

The relevance of central tolerance at this initial checkpoint in the pathogenesis of autoimmunity has gained credence in the last 10 years after the demonstration that many tissue-restricted Ags, including TSHR and insulin, are present in the thymus, and that their expression is important to prevent autoimmunity (13). This phenomenon, designated by Kyewsky as “promiscuous gene expression,” is dependent, in part, on the autoimmune regulator (AIRE) gene (13, 14). This implication of central tolerance in the prevention of tissue-specific autoimmune disease is supported by the demonstration that in the rare recessive genetic disease autoimmune polyendocrine syndrome, the inactivation of the AIRE gene leads to the loss of thymic expression of peripheral Ags and to multiple organ autoimmune diseases, including AITD, in affected individuals (15). Mice in which the AIRE gene is inactivated also show strain-dependent multiorgan autoimmunity (16, 17).

As for most autoimmune diseases, the etiopathogenesis of AITD remains incompletely understood, but twins and family studies have determined that genetic factors play a major role. Gene linkage and association studies, including genome-wide association studies, have identified the following predisposing genes, listed in descending order of strength of association: HLA, TSHR, CTLA4, PTPN22, CD40, CD25, and Tg (18). However, in most cases, the mechanism through which these predisposing genes confer susceptibility to disease remains unclear. For the TSHR
gene we have proposed a plausible mechanism for its contribution to pathogenesis (19). In brief, as we have demonstrated that the protective allele determines a higher level of TSHR in the thymus, we postulated that this higher level would result in a more efficient negative selection of TSHR-reactive thymocytes during maturation, and therefore in a peripheral T lymphocyte repertoire more tolerant to TSHR. This would reduce the induction of an anti-TSHR response through cross-reaction with pathogens (20, 21).

In the course of the earlier experiments, we detected, rather unexpectedly, that in the thymus gland, TSHR is expressed in thymocytes at a higher level than in the medullary epithelial cells, the cells where most peripheral tissue–restricted Abs involved in the induction of central tolerance are expressed (22). Overall, TSHR expression in the thymus is presumably more than sufficient to supply Ag to APCs, which can then, in turn, present it to immature thymocytes and induce deletion of TSHR-specific, TCR-bearing thymocytes. However, the role of TSHR expressed in thymocytes remains unexplained. Of interest, thymic hyperplasia, even if not widely appreciated, is a well-established feature of GD that could be explained by the continuous action of TSAbs on maturing thymocytes expressing TSHR. This has been previously proposed on the basis of the expression of TSHR in total thymic tissue and on clinical observations (23, 24).

One interesting possibility is that the action of TSAbs on thymocytes would favor the escape of autoreactive T cells from the thymus. This possibility is suggested by the recent publication from van der Weerd et al. (25) showing that TSHR in thymocytes is functional and signals in response to TSH. From all the above, it is clear that elucidating whether TSHR in thymocytes could be activated by TSAbs is crucial to better understand the physiological role of TSHR in thymocytes and how it may contribute to the pathogenesis of GD. To this end, we set up experiments aimed at: 1) confirming the expression of TSHR in thymocytes, 2) determining the stage of maturation at which it is expressed, and 3) demonstrating that both TSH and TSAbs from GD patients can indeed induce TSHR-mediated signals in human thymocytes. In this article, we present results that support the view that TSAbs not only stimulate thyroid follicular cells, but also influence thymocyte maturation, and thus may contribute to maintain and focus the centrally tolerized T cells where most peripheral tissue–restricted Ags involved in the induction of central tolerance are expressed (22). Overall, TSHR expression in the thymus is presumably more than sufficient to supply Ag to APCs, which can then, in turn, present it to immature thymocytes and induce deletion of TSHR-specific, TCR-bearing thymocytes. However, the role of TSHR expressed in thymocytes remains unexplained. Of interest, thymic hyperplasia, even if not widely appreciated, is a well-established feature of GD that could be explained by the continuous action of TSAbs on maturing thymocytes expressing TSHR. This has been previously proposed on the basis of the expression of TSHR in total thymic tissue and on clinical observations (23, 24).

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Materials and Methods

Tissue processing and cell culture

Thymic samples were obtained with informed parental consent from patients undergoing corrective cardiac surgery (age, 4 mo to 13 y). They belong to our thymus biobank collection that was set up in 2006 to contribute to research in autoimmune diseases. Donors with autoimmune or autoimmune-related diseases were excluded. All samples were processed within 4 h of resection under sterile conditions as previously described (26). In brief, thymuses were finely minced and gently stirred in a screw-capped Erlenmeyer flask in cold RPMI 1640 (Sigma, Deisenhofen, Germany) with 10% FCS and 50 U/ml penicillin, 10 mg/ml streptomycin, and 50 U/ml hygromycin (Thermo Fisher Scientific).

Protein extracts preparation and protein immunoblotting

Thyroid tissue from three adult GD patients and thymus tissue from seven pediatric patients were cut into 0.5-cm pieces and homogenized using Polytron in 5 vol radioimmunoprecipitation assay buffer (Thermo Fisher Scientific) supplemented with a combination of protease inhibitors (Thermo Fisher Scientific) and 1 mM PMSF (Sigma). HEK-TSHR and thymocyte total protein extracts were obtained by in-plate lysis of the cells plated in 10-cm petri dishes or by lysis of the pelleted cells, respectively, using the same lysis buffer as described earlier. Homogenized tissue, pelleted thymocytes, and HEK-TSHR cells were incubated in the lysis buffer on ice for 30 min and then centrifuged at 20,000 x g at 4˚C for 30 min. Clarified lysates were transferred into fresh tubes and the protein concentration measured by the BCA method (Thermo Fisher Scientific). Cell lysates (20 mg protein equivalents) were separated by electrophoresis on 10% SDS-PAGE under denaturing and reducing conditions, then electrophoretically transferred onto nitrocellulose membranes (Invitrogen).

Membranes were then blocked with 5% milk in PBS containing 0.1% Tween 20 and then incubated with primary mouse monoclonal anti-human TSHR Ab A10 (raised against residues 21–35; Abcam) at a 1/10,000 dilution. Parallel control membranes with the primary Ab replaced by PBS or isotype-matched, nonimmune Igs (Sigma, Deisenhofen, Germany) were also processed to confirm specificity and to exclude cross-reactivity. As a loading control, a mouse mAb against b-actin (Sigma) followed by a secondary goat anti-mouse HRP conjugate (Invi- trogen) was used. Membranes were developed with West Pico chemiluminescent substrate (Thermo Fisher Scientific).

TSHR mRNA quantification

Relative TSHR expression quantification was assessed by quantitative PCR using the TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer’s protocol and normalized by GAPDH expression. Reactions were run on a LightCycler 480 instrument (Roche) in triplicate, and the average cycle threshold value of the triplicates was used for further statistical analysis (28).

Cell sorting of thymocytes

Total thymocytes obtained as described earlier were stained with the combination of Abs shown in Table I and sorted in Becton and Dickinson Aria cell sorter in the Flow Cytometry facility of the Institut d’Investigació en Ciències de la Salut Germans Trias i Pujol (Badalona, Spain) to isolate the cell populations listed in Table I.

Antibodies

Human mAbs to the TSHR with stimulating (M22) and blocking (KI-70) activities were purchased from RSR Limited (Cardiff, U.K.). We also used a normal IgG preparation from Caltag Laboratories. Igs from TSAbs-positive and control patient sera were isolated using MAbTrap GII (GE Healthcare Life Sciences) following the manufacturers’ instructions and adjusted to 1 mg/ml.

Measurement of cAMP production in cell cultures

Unfractionated thymocytes were freshly seeded into 24-well plates at a density of 1 x 10^6 cells/well in HBSS with 10 mM HEPES and 1% BSA without 3-isobutyl-1-methylxanthine (IBMX; Sigma) for 2 h before functional analyses. Thymocytes were then washed and incubated for 1 h in HBSS with 10 mM HEPES and 1% BSA in the presence of 1 mM IBMX. Increasing concentrations of the following stimuli were added and incubated for 1 h: Forskolin, bovine TSH (bTSH), recombinant human TSH (rTSH), mAbs, human sera, and purified human IgGs. Human primary thyrocytes and HEK-TSHR cells were seeded the day before the experiment at a density of 2 x 10^5 cells/well in DMEM containing 10% FBS for thyrocytes and the same medium supplemented with 100 U/ml penicillin, 10 mg/ml streptomycin, and 50 U/ml hygromycin for HEK-TSHR. After overnight culture, cells were washed three times and the different stimuli indicated earlier were added and incubated for 1 h. To assess basal cAMP production, we incubated thyrocytes and thymocytes for 1 h at 37˚C in a humidified incubator in HBSS with 10 mM HEPES...
(supplemented with 1% BSA for thymocytes) containing only 1 mM IBMX, as described by Neumann et al. (27). After incubation, cells were lysed using lysis buffer, and cAMP content of the cell lysate was measured using the cAMP-Screen Direct system (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions.

**Measurement of TSAb activity in human IgGs using JPO9 cells**

JPO9 cells (CHO cells expressing TSHR) were seeded at the concentration of 25,000 cells/well into 96-well plates with DMEM with 10% FBS and incubated overnight at 37°C in a humidified 5% CO2 incubator. Cells were then washed with HBSS and incubated for 1 h at 37°C. Cells were stimulated with various concentrations of purified IgGs from GD patients and from healthy control subjects. Assays were performed in triplicate for each experiment. Extracellular cAMP accumulation in the medium was determined using an in-house radioimmunoassay method using a commercial polyclonal anti-CAMP Ab, as previously described (29). Results were expressed as the fold-increase of cAMP released in the extracellular medium over the basal value.

**Blocking TSHR stimulation in primary human thymocytes**

Specific inhibition of the stimulatory effect of bTSH (10 mIU/ml, final concentration) and IgG preparations (100 µg/ml, final concentration) was assessed by measuring cAMP in culture replicates preincubated with increasing dilutions of the blocking mAb K1-70 (from 0.1 to 100 µg/ml final concentration).

**Statistical analysis**

Intracellular cAMP levels obtained after induction with the different stimuli were compared with their respective basal levels or to control samples using the Mann–Whitney U test to calculate two-tailed exact p values. Because cAMP values were not normally distributed, nonparametric tests were applied. Correlations between the TSAb activities of IgG preparations measured in the JPO9 cell line and the cAMP levels measured in thymocytes were analyzed using nonparametric Spearman correlation analysis. Statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Statistical significance was set at p < 0.05.

**Results**

**Demonstration of TSHR protein expression in the thymus**

TSHR mRNA and protein expression in the thymus was investigated. Full-length hTSHR mRNA expression in thymocytes was demonstrated by PCR and sequencing (data not shown). To investigate TSHR protein expression, we tested several Abs directed to the TSHR-A and -B subunits. Only the A10 mAb raised against the N terminus (residues 21–35) of the TSHR gave clear results. To investigate TSHR protein expression, we tested several Abs directed to the TSHR-A and -B subunits. Only the A10 mAb raised against the N terminus (residues 21–35) of the TSHR gave clear results.

**Intracellular cAMP levels obtained after induction with the different stimuli**

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**TSHR highly expressed in immature thymocytes, but not in mature thymocytes and naïve T cells**

In a previous study, we provided the first evidence that TSHR mRNA was expressed in thyrocytes, both in CD2+CD3- and in CD2+CD3+ subsets (19). In this study, we determined the stages of thymic maturation at which it is expressed. For this purpose, we purified the following thymic populations (Table I) from pediatric donors: 1) early thymic progenitors (ETP): CD34+CD1A-CD4--; 2) pre-T cells (committed to the T cell lineage) (30): CD4+CD1a+CD4--; 3) double-positive (DP) cells: CD3+CD4+CD8--; 4) single-positive CD4+ cells (SP4): CD3+CD4+CD8--; and 5) single-positive CD8+ cells (SP8): CD3-CD4-CD8+. We measured TSHR mRNA expression by quantitative PCR and found that the most immature populations (ETP and pre-T) already express significant amounts of TSHR, and its expression increases with maturation, reaching a maximum level in DP cells (Fig. 2). In contrast, in single-positive cells, the TSHR expression is very low: SP4 and SP8 expressed 45-fold and 118-fold lower RNA levels, respectively, compared with DP cells. To determine whether TSHR was expressed by peripheral T cells, we purified naïve and memory CD4 and CD8 T cells (see Materials and Methods for the lineage markers used). Within the naïve population, we also purified the CD3+CD4+ subset to enrich in recent thymic emigrants. We did not find TSHR mRNA expression in any of these peripheral populations. We also purified CD4+ and CD8+ T cells from cord blood because these cells are, by definition, highly enriched in recent thymic emigrants. Again, the TSHR mRNA was not expressed. Collectively, these results indicate that TSHR is expressed at all stages of immature thymocytes (from ETP to DP cells), residually expressed in mature single-positive thyrocytes, and not expressed in extrathymic T cell populations.

**Human thymocytes express functional TSHR**

In thyroid cells, cAMP is a second messenger generated by the activation adenylate cyclase (AC) due to TSHR stimulation. It is commonly used as a readout for the efficacy of TSHR ligands (27). To investigate whether TSHR in human thyrocytes is functional, we tested thyrocytes for their ability to generate cAMP in re-

**FIGURE 1.** TSHR protein is expressed in thyrocytes. Each lane contains 20 µg protein extract from the indicated cells: HeLa (negative control), primary thyrocytes and HEK-TSHR (positive controls), as well isolated thyrocytes (TMB 1 to TMB 6 lanes). TSHR species were detected with A10 primary mAb directed against the ectodomain (residues 21–35). A β-actin mAb was used as a loading control. Expected m.w. of TSHR holoreceptor and TSHR A-subunit, as well as β-actin, are indicated to the right of the figure. The vertical line is to indicate where a lane was removed from the Western blot image and separate parts of the image were joined together. All samples were run and probed at the same time with the corresponding Abs.
Table I. Surface markers used in thymocyte cell sorting

<table>
<thead>
<tr>
<th>Population</th>
<th>Surface Markers Used in Cell Sorting</th>
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<tbody>
<tr>
<td>Thymic populations</td>
<td>CD3+CD1A+CD45acCD4+</td>
</tr>
<tr>
<td>ETP</td>
<td>CD3+CD1A+CD45acCD4+</td>
</tr>
<tr>
<td>Pre-T</td>
<td>CD3+CD4+CD8+</td>
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<tr>
<td>DP</td>
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<td>T CD8 memory</td>
<td>CD3+CD4+CD5RA+CD62L+CD31+</td>
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sponse to different stimuli. In all experiments, 1 mM IBMX, a well-known phosphodiesterase inhibitor, was added to the medium. Forskolin, an AC agonist, provided the positive control that induced a robust 15-fold increase in intracellular cAMP over basal levels, with a good dose–response curve (Fig. 3A). bTSH elicited a moderate but significant increase of cAMP above basal levels over the range of concentrations tested (1–50 mIU/ml; Fig. 3B). We observed a reproducible bell-shaped response, with the highest response at 10 mIU/ml, and a decrease at 50 mIU/ml, similar to that observed in the HEK-TSHR cell line (31). This increase was stable for up to 6 h (Fig. 3C). We also evaluated the ability of rhTSH to stimulate cAMP production in thymocytes. Fig. 3D shows that the activity of rhTSH was statistically equivalent to that of bTSH, with the highest increase in intracellular cAMP levels in thymocytes. Fig. 3C shows that the activity of rhTSH (Fig. 3C). We also evaluated the ability of rhTSH to stimulate cAMP production in thymocytes. Fig. 3D shows that the activity of rhTSH was statistically equivalent to that of bTSH, with the highest increase in cAMP levels above basal levels at 10 mIU/ml (3.52 ± 0.68, p = 0.0211 and 2.970 ± 0.43, p = 0.0214, respectively, compared with Fig. 3B). As further cellular substrate positive controls, we measured intracellular cAMP levels upon stimulation with bTSH in the HEK-TSHR cell line (Fig. 3E) and in human primary thyrocyte cultures (Fig. 3F). In both cases, 10 mIU/ml bTSH induced a robust increase in intracellular cAMP levels, corresponding to 130- and 11-fold compared with basal levels, respectively.

TSHR-stimulating, but not TSHR-blocking, mAbs to TSHR stimulate cAMP production in thymocytes

To investigate the ability of human thymocytes to signal through TSHR, we used the TSHR-specific mAbs M22 and K1-70. These Abs were originally obtained from thyroid autoimmune patients and are representative of TSHR Abs in GD, stimulating or blocking (respectively) TSHR autoantibodies (32, 33). The stimulating mAb M22 was capable of inducing a significant increase above basal levels in human thymocytes (Fig. 4A), human primary thyrocytes (Fig. 4B), and HEK-TSHR cells (Fig. 4C). It is clear that thymocytes are responsive but less sensitive than the other two cell substrates. This finding is consistent with greater concentrations of TSHR in thyrocytes, and especially in HEK-TSHR cells. As expected from its well-known blocking nature, K1-70, as well as an irrelevant control IgG, did not induce intracellular cAMP levels greater than basal levels in the range of concentrations tested in any of the cellular substrates used (Fig. 4).

Sera and purified IgGs from patients with GD stimulate cAMP production in thymocytes

To test the ability of TSAbs from GD patients to induce cAMP in thymocytes, we first tested sera from four patients using the same HEK-TSHR tissue culture system as described earlier. Two patients out of four induced a significant 2- and 3-fold increase of cAMP over basal levels (2.130 ± 0.066 pmol/well, p = 0.0216 and 3.027 ± 0.255 pmol/ml, p = 0.0309). To confirm that this effect was mediated by TSAbs, we tested a series of purified IgG preparations from 17 active GD patients and from 17 healthy individuals in thyrocyte cultures from two different glands in triplicate cultures. Overall, purified IgGs from GD patients significantly stimulated cAMP production above basal levels in human thymocytes at all concentrations tested, whereas IgG preparations from healthy individuals did not have any significant effect (Fig. 5A and 5B, respectively). Not unexpectedly, each IgG preparation had a different dose response, but at 40 μg/ml virtually all (94%) IgG preparations from GD patients significantly stimulated intracellular cAMP above basal levels. These results clearly show that thymocytes are responsive to the TSAbs present in the sera of GD patients. We found a good correlation between TSAb activity measured using the JPO9 cell assay (Table II) and activity of these IgG preparations in thymocytes (Table III). The positive correlation was clear at the range of con-
concentrations from 0.4 to 40 μg/ml (Table III). At 160 μg/ml, the correlation was negative, in agreement with the desensitization of the TSHR at high concentrations already observed throughout this study and as previously described (31). Overall, this indicates that IgGs from GD patients with high TSAb activities are clear activators of thymocyte TSHR.

To confirm the specificity of the thymic TSHR, we determined whether the blocking K1-70 mAb was able to compete with other TSHR ligands in purified human thymocytes. Fig. 5C shows that K1-70 blocks in a similar dose-dependent manner the stimulatory activity of both bTSH and the four purified IgG preparations tested. The effect was maximal at 400 μg/ml K1-70. These find-
ings suggest that thymic TSHR behaves in a manner similar to that observed in thyrocytes and in the cell lines expressing the human TSH receptor.

**Discussion**

In this article, we provide evidence that TSAbs that occur in GD patients can stimulate thymocytes through the TSHR. The stimulation of TSHR may explain GD-associated thymus hyperplasia and, even more interestingly, could also influence the autoimmune response to the TSHR.

Expression of TSHR in thymocytes was reported by our group in 2011 (19) and was confirmed by van der Weerd et al. (25). We have expanded these results by showing that TSHR message and proteins are expressed normally in thymocytes, and found that the main isoform in thymocytes coincides in m.w. and sequence with the main isoform expressed by thyroid follicular cells. A rather complex mix of mRNAs corresponding to minor transcripts was detected in thy-

mocytes; it is currently being analyzed and will be reported separately (Marín-Sánchez et al., manuscript in preparation). Interestingly, TSHR is expressed in thymocytes from an early stage of differenti-

ation but disappears completely after cells leave the thymus, sug-

gesting a role in maturation that is confined to the thymus (25).

We have shown that TSHR in thymocytes is functional, because cAMP responses were elicited by bTSH. Most importantly, we showed that sera and purified IgGs with TSAb activity from GD patients were able to induce cAMP production in a dose-dependent manner on freshly isolated human thymocytes. The HEK-TSHR transfected cell line and the M22 mAb that faithfully reproduces the action of patients’ TSAbs have been used as control cell substrate and positive stimulus, respectively. The precise role for the TSHR in thymocyte biology will require further investigations.

We propose two possible contributions of TSAb interactions with TSHR on thymocytes: 1) participation in thymus hyperplasia in GD; and 2) creation of a vicious cycle of iterative improvement of the affinity and stimulating capability of an initially antibacterial Ab cross-reactive with the TSHR, leading to development of more and more potent autoantibodies. Both of these hypotheses are discussed in the following paragraphs.

**FIGURE 4.** TSHR-stimulating, but not blocking, mAbs to the TSHR induce cAMP production in human thymocytes. Intracellular cAMP was measured by ELISA after 1-h incubation with the indicated amounts of the mAb with stimulating activity M22 (dotted line), the monoclonal thyroid-blocking Ab K1-70 (solid line), or a normal mouse IgG (dashed line) in (A) human thymocytes, and in substrate controls (B) human primary thyrocytes, and (C) the HEK-TSHR cell line. Bars represent mean ± SEM of three to four experiments. All experiments are performed in triplicates; *p < 0.05.

**FIGURE 5.** Purified IgG preparations from GD patients can stimulate thymic TSHR. Intracellular cAMP of human thymocytes was measured by ELISA after 1-h incubation with the indicated amounts of purified IgGs from (A) 17 active GD patients or (B) 17 healthy control subjects tested in 2 thymus glands and in triplicates. Bars represent mean ± SEM of two experiments. (C) Inhibitory effect of K1-70 blocking mAb on cAMP stimulation in primary human thyrocytes by bTSH (10 mIU/ml final concentration, open circle) and four IgG preparations from four GD patients: GDT1 (open square), GDT13 (filled square), GDT14 (filled triangle), and GDT17 (filled inverted triangle) all at 100 µg/ml, final concentration. Mean of triplicate determinations. Bars rep-
The mechanism behind thymic hyperplasia in GD has been the subject of speculation. Since the detection up to 1996 of TSHR in total thymus tissue extracts (23, 34), the possibility of TSAbs being involved in the process has been repeatedly proposed (35), and a possible effect of GD IgGs on thymocytes was first experimentally demonstrated, in a single case, in 1988 (36). Our data demonstrating the functional interaction between TSAbs and thymic TSHR supports this hypothesis. In GD, TSH levels are low, demonstrating the functional interaction between TSAbs and the TSHR Abs can therefore be envisaged to occur first in the lymph nodes and then later in the diffuse lymphoid organ hyperplasia detected in GD patients. The diffuse lymphoid organ hyperplasia will increase clinical awareness on the fact that recognizing the TSHR. When these Abs reach the thymus, they will inevitably provide help for the production of Abs reactive anti-TSHR Abs produced in the peripheral lymphoid organs, where centroblasts divide, accumulate somatic mutations, and switch Ig class. The driving interactions are those with the Ag retained on the surface of follicular dendritic cells and with the T follicular helper cells in the GCs milieu (41). If cross-reactive anti-TSHR Abs produced in the peripheral lymphoid organs reach the thymus, they would stimulate the maturation of TSHR-expressing thymocytes and increase the rate at which naive T cells leave the thymus. Because naive T lymphocytes circulate preferentially through the lymph nodes and home in to active GCs (42), some of these naive T cells will likely home in to the GCs that are generating Abs cross-reactive with TSHR. Inevitably, a very small but not negligible proportion of these naive T cells will be able to recognize TSHR-like peptides presented by B cells in these GCs and would provide help for the production of Abs that recognize the TSHR. When these Abs reach the thymus, they will further increase the release of T cells, some of which again would inevitably provide help for the autoimmune response to the TSHR. This iterative process could soon also involve the cervical lymph nodes draining the thyroid, rich in TSHR and often activated by oropharyngeal infections (43). This would result in the production of high-affinity stimulating TSHR Abs, namely, the TSAbs themselves with the immature TSHR-expressing thymocytes (see later).

As it is well-known, the fine-tuning of the Ab response to increase affinity and specificity takes place in the GC of secondary lymphoid organs, where centroblasts divide, accumulate somatic mutations, and switch Ig class. The driving interactions are those with the Ag retained on the surface of follicular dendritic cells and with the T follicular helper cells in the GCs milieu (41). If cross-reactive anti-TSHR Abs produced in the peripheral lymphoid organs reach the thymus, they would stimulate the maturation of TSHR-expressing thymocytes and increase the rate at which naive T cells leave the thymus. Because naive T lymphocytes circulate preferentially through the lymph nodes and home in to active GCs (42), some of these naive T cells will likely home in to the GCs that are generating Abs cross-reactive with TSHR. Inevitably, a very small but not negligible proportion of these naive T cells will be able to recognize TSHR-like peptides presented by B cells in these GCs and would provide help for the production of Abs that recognize the TSHR. When these Abs reach the thymus, they will further increase the release of T cells, some of which again would inevitably provide help for the autoimmune response to the TSHR. This iterative process could soon also involve the cervical lymph nodes draining the thyroid, rich in TSHR and often activated by oropharyngeal infections (43). This would result in the production of high-affinity stimulating TSHR Abs, namely, the TSAbs typical of GD. Of note, an excess of naive T cells has also been detected among intrathyroidal lymphocytes in GD patients, consistent with our hypothesis (44). Obviously all active B cells, not only the THSR-specific ones, would receive a boost from this increase in the availability of naive T cells, and this may explain the diffuse lymphoid organ hyperplasia detected in GD patients.

A vicious cycle that leads to the continuous improvement of the affinity and stimulating-capability of the TSHR Abs can therefore be envisaged to occur first in the lymph nodes and then later in the

Table III. Correlations between intracellular cAMP levels induced in thymocytes by purified IgGs from GD and healthy patients with their respective TSAb activities measured in JPO9 cells at each of the indicated concentrations tested

<table>
<thead>
<tr>
<th>Final Concentration of IgGs Tested (µg/ml)</th>
<th>GD Patients</th>
<th>Healthy Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^a$</td>
<td>$p^b$</td>
</tr>
<tr>
<td>0.4</td>
<td>0.6724</td>
<td>0.0003</td>
</tr>
<tr>
<td>4</td>
<td>0.7408</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>40</td>
<td>0.4916</td>
<td>0.0147</td>
</tr>
<tr>
<td>160</td>
<td>-0.4518</td>
<td>0.0267</td>
</tr>
</tbody>
</table>

$^a$Spearman $r$ correlation values.

$^b$Two-tailed $p$ value. All values calculated taking into account all IgGs from each group at each of the concentrations indicated.
thyroid gland itself. Experiments are required to demonstrate that TSAbs do indeed enhance the release of autoreactive T cells from the thymus, and that these cells are important for the affinity maturation of TSHR-specific B cells in the periphery. Some of these are not feasible in humans and will need to take advantage of some of the new models of GD in mice.

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Disclosures

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