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# *Schistosoma mansoni* and $\alpha$ -Galactosylceramide: Prophylactic Effect of Th1 Immune Suppression in a Mouse Model of Graves' Hyperthyroidism

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Graves' hyperthyroidism, an organ-specific autoimmune disease mediated by stimulatory thyrotropin receptor (TSHR) autoantibodies, has been considered a Th2-dominant disease. However, recent data with mouse Graves' models are conflicting. For example, we recently demonstrated that injection of BALB/c mice with adenovirus coding the TSHR induced Graves' hyperthyroidism characterized by mixed Th1 and Th2 immune responses against the TSHR, and that transient coexpression of the Th2 cytokine IL-4 by adenovirus skewed Ag-specific immune response toward Th2 and suppressed disease induction. To gain further insight into the relationship between immune polarization and Graves' disease, we evaluated the effect of Th2 immune polarization by helminth *Schistosoma mansoni* infection and  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), both known to bias the systemic immune response to Th2, on Graves' disease. *S. mansoni* infection first induced mixed Th1 and Th2 immune responses to soluble worm Ags, followed by a Th2 response to soluble egg Ags. Prior infection with *S. mansoni* suppressed the Th1-type anti-TSHR immune response, as demonstrated by impaired Ag-specific IFN- $\gamma$  secretion of splenocytes and decreased titers of IgG2a subclass anti-TSHR Abs, and also prevented disease development. Similarly,  $\alpha$ -GalCer suppressed Ag-specific splenocyte secretion of IFN- $\gamma$  and prevented disease induction. However, once the anti-TSHR immune response was fully induced, *S. mansoni* or  $\alpha$ -GalCer was ineffective in curing disease. These data support the Th1 theory in Graves' disease and indicate that suppression of the Th1-type immune response at the time of Ag priming may be crucial for inhibiting the pathogenic anti-TSHR immune response. *The Journal of Immunology*, 2004, 173: 2167–2173.

Graves' disease is an organ-specific autoimmune disease in which stimulatory thyrotropin receptor (TSHR)<sup>2</sup> autoantibodies (thyroid-stimulating Abs (TSAb)) cause hyperthyroidism and diffuse goiter (1). Understanding the pathogenesis of Graves' disease has been aided by the development of animal models. For instance, in our mouse model involving repeated i.m. injection of adenovirus coding the TSHR (Ad-TSHR) (2), the TSHR Abs induced are of both IgG1 and IgG2a subclasses, and splenocytes produce the Th1 cytokine IFN- $\gamma$  in response to in vitro stimulation with TSHR Ag (3). Similarly, Prabhakar et al. (4) have found both IgG1 and IgG2a of TSHR Abs and Ag-specific splenocyte secretion of IFN- $\gamma$  and the Th2 cytokine IL-4 after injecting M12 B lymphoma cells expressing the TSHR. These data indicate the involvement of both Th1 and Th2 immune responses in the pathogenesis of Graves' disease, at least in

animal models. Furthermore, we have also shown that transient coexpression of TSHR and IL-4 by adenovirus induces Th2 deviation of the anti-TSHR immune response and suppresses disease induction. In contrast, coinjection of adenovirus coding IL-12 deviates the anti-TSHR immune response to Th1 without affecting disease incidence (3). Thus, Th2 polarization may be beneficial for the control of Graves' disease. However, because of the transient nature of transgene expression by adenovirus, our results imply the suppressive effect of transiently overexpressed exogenous IL-4 on disease development. We were curious to know the effect of more physiological, long-lasting Th2 immune deviation on Graves' disease. We therefore evaluated in this study the effect of Th2 immune deviation by  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) and infection with helminth *Schistosoma mansoni* on Graves' disease in our mouse model.

$\alpha$ -GalCer, a marine-sponge derived glycolipid, is an agonistic ligand for invariant NKT cells (5). NKT cells represent a unique immune cell lineage that have characteristics of both NK and T cells, expressing NK cell markers such as NK1.1 and a highly restricted TCR repertoire (V $\alpha$ 14J $\alpha$ 281V $\beta$ 8.2 in mice and V $\alpha$ 24J $\alpha$ QV $\beta$ 11 in humans) (6, 7). These cells have a unique Ag recognition system, in that they recognize glycolipid Ags in conjunction with the nonpolymorphic, nonclassical major MHC class I-like molecule CD1d expressed on most hemopoietic cells. Although a natural ligand(s) for CD1d has not yet been identified,  $\alpha$ -GalCer has recently been shown to be a potent stimulator of both human and mouse NKT cells (5).  $\alpha$ -GalCer stimulates NKT cells to rapidly produce both Th1 and Th2 cytokines, but at a later time point and with repeated doses,  $\alpha$ -GalCer promotes the development of a Th2 immune response (8, 9). NKT cells regulate numerous immune responses during innate and adaptive immunities and are reported to influence the pathogenesis of Th1-dominant

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<sup>2</sup> Abbreviations used in this paper: TSHR, thyrotropin receptor; TSAb, thyroid-stimulating Ab; Ad-TSHR, adenovirus expressing the human TSHR; p.i., postinfection;  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; EAE, experimental autoimmune encephalitis; TBIAb, TSH binding-inhibiting Ab; SWA, soluble worm Ag; SEA, soluble egg Ag; T<sub>4</sub>, thyroxine.

autoimmune diseases. For example, a reduced number and/or impaired function of NKT cells are demonstrated in several autoimmune diseases, such as type I diabetes, experimental autoimmune encephalitis (EAE)/multiple sclerosis, and lupus (10–16). Passive transfer of NKT cells or transgenic expression of V $\alpha$ 14J $\alpha$ 281 have previously been shown to suppress diabetes or EAE (17, 18). Prevention and/or cure of diabetes, EAE, and colitis with  $\alpha$ -GalCer have also been demonstrated in mouse models (19–27). Conversely, CD1d-deficient NOD mice have an earlier disease onset, increased disease penetrance, and more severe disease (22, 23, 28).

Helminth infections have potent systemic immunomodulatory effects on the host immune system directed to other infectious agents and noninfectious Ags (29). For example, *S. mansoni* infection is initiated by penetration of the skin by waterborne cercariae that migrate into the bloodstream and finally arrive in the hepatic portal system, where the differentiated male and female mate. At approximately the fifth week postinfection (p.i.), the mature female worms begin to produce large numbers of eggs, which lodge in the liver and intestinal tissue where they induce a granulomatous response that results in the major pathologic manifestations of the disease (30). The immune response to *S. mansoni* is reportedly initially a Th1 phenotype against the larval and adult worms, followed by a Th2 response to the parasite eggs 6–8 wk p.i. (31, 32). Thus, a sustained Th2-dominant immune response to *S. mansoni* egg Ags probably creates a cytokine environment that induces a Th1 to Th2 shift of the immune responses to unrelated Ags (33–35). In addition, of interest, *S. mansoni* eggs and worms express a variety of glycoconjugates, including GalCer (36), suggesting that the effect of *S. mansoni* infection may at least partly be mediated by NKT cells. *S. mansoni* infection has been shown to suppress Th1 autoimmune diseases, including type 1 diabetes (37, 38), EAE (39), and arthritis (40).

We show in this study that suppression of the Th1-type anti-TSHR immune response by prior infection with *S. mansoni* or  $\alpha$ -GalCer at the time of Ag priming prevents the development of Graves' hyperthyroidism, but after activation of an anti-TSHR immune response cannot cure disease, indicating a difficulty in reverting an already established pathogenic anti-TSHR immune response by such immunological interventions.

## Materials and Methods

### Immunization with Ad-TSHR

Nonreplicative recombinant adenovirus expressing the human TSHR (Ad-TSHR) has previously been described (2). Amplification, purification, and determination of the viral particle concentration were performed as previously described (2). Female BALB/c mice (6 wk old; Charles River Laboratories, Tokyo, Japan) were injected in the quadriceps with 50  $\mu$ l of PBS containing 10<sup>11</sup> particles of Ad-TSHR three times at 3-wk intervals as previously described (2). Blood was drawn at the time points indicated. All experiments were conducted in accordance with the principles and procedures outlined in the Guidelines for the Care and Use of Laboratory Animals at Nagasaki University. Mice were kept under specific pathogen-free conditions through the experiments.

### $\alpha$ -GalCer treatment

$\alpha$ -GalCer was provided by Kirin Brewery (Gunma, Japan) and was dissolved in a vehicle of H<sub>2</sub>O containing 0.05% polysorbate 20 (5). In a prophylactic setting, mice were injected i.p. with 100  $\mu$ g/kg (~2  $\mu$ g/mouse)  $\alpha$ -GalCer or vehicle at the time of each immunization and on days 3 and 7 postimmunization. In a therapeutic setting, i.p. injection of  $\alpha$ -GalCer (300  $\mu$ g/kg; ~6  $\mu$ g/mouse, twice a week) was initiated 1 wk after the third immunization with Ad-TSHR and was continued for 7 wk.

### Infection with *S. mansoni* and immunization with soluble worm Ag (SWA) and soluble egg Ag (SEA) proteins

The maintenance and production of *S. mansoni* have been previously described (41). SWA and SEA proteins were prepared from homogenized

adult worms and eggs, respectively, using previously described procedures (42, 43). Mice were infected with *S. mansoni* by percutaneous exposure of the abdomen to 15 cercariae. Successful infection of *S. mansoni* was monitored by ELISA against SWA and SEA proteins (see below). Alternatively, mice were i.p. injected with 50  $\mu$ g/mouse of SWA or SEA proteins on days -7, -4, and 0 of each immunization with AdTSHR.

### Thyroxine (T<sub>4</sub>), TSAb, and TSH binding inhibiting Ab (TBIAb) measurements

Total serum T<sub>4</sub> was measured with an RIA kit (RIA-gnost tT<sub>4</sub>; Nippon Schering, Osaka, Japan). The normal range was defined as the mean  $\pm$  3 SD of control untreated mice. TSAb activities in mouse sera were measured with FRTL5 cells (2). The cells seeded at 3  $\times$  10<sup>4</sup> cells/well in a 96-well plate were incubated in 50  $\mu$ l of hypotonic HBSS containing 0.5 mM isobutylmethylxanthine, 20 mM HEPES, 0.25% BSA, and 5  $\mu$ l of serum for 2 h at 37 C. cAMP released into the medium was measured with a cAMP RIA kit (Yamasa, Tokyo, Japan). A value >150% of that in control mice was judged positive. TBIAb values were determined with a TRAb kit (BRAHMS Diagnostica, Berlin, Germany) using 10  $\mu$ l of serum. A value >15% inhibition of control binding was judged positive.

### ELISA for Abs to TSHR, SWA, and SEA

ELISA wells were coated overnight with 100  $\mu$ l of TSHR-289 protein (1  $\mu$ g/ml) (44), SEA (10  $\mu$ g/ml), or SWA (10  $\mu$ g/ml) and incubated with mouse serum (1/100 to 1/1000 dilutions). After incubation with HRP-conjugated anti-mouse IgG (diluted 3000-fold; A3673; Sigma-Aldrich, St. Louis, MO), or subclass-specific anti-mouse IgG1 and IgG2a (diluted 1000- and 1500-fold; X56 and R19-15; BD Pharmingen, San Diego, CA), color was developed using orthophenylene diamine and H<sub>2</sub>O<sub>2</sub> as substrate, and OD was read at 492 nm.

### Cytokine assays

Splenocytes were cultured (triplicate aliquots) at 5  $\times$  10<sup>5</sup> cells/well in a 96-well, round-bottom plate in the presence or the absence of TSHR-289 protein (5  $\mu$ g/ml),  $\alpha$ -GalCer (50 ng/ml), or Con A (5  $\mu$ g/ml). Five days later, the concentrations of IFN- $\gamma$ , IL-4, and IL-10 in the medium were determined with ELISA kits (BioSource International, Camarillo, CA). Cytokine production was expressed as picograms per milliliter using standard curves of recombinant mouse cytokines.

### Statistical analysis

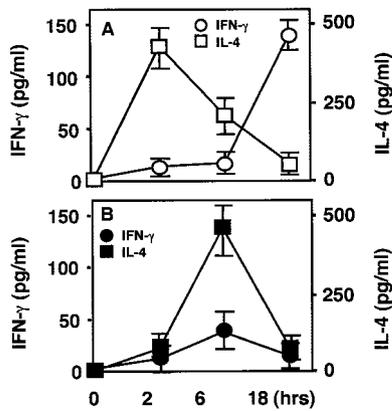
Levels of Abs and cytokines were analyzed by Mann-Whitney *U* test. The incidence of disease was evaluated by  $\chi^2$  test. A value of *p* < 0.05 was considered significant.

## Results

### Effect of $\alpha$ -GalCer on Graves' disease induction

We first tested the ability of  $\alpha$ -GalCer to stimulate NKT cells in BALB/c mice to produce cytokines and to modulate adaptive immune responses. A single i.p. injection of 100  $\mu$ g/kg  $\alpha$ -GalCer induced a rapid increase in serum IL-4 and a delayed elevation in IFN- $\gamma$  (Fig. 1A). However, prior in vivo injections of  $\alpha$ -GalCer on days -10, -7, and -3 suppressed  $\alpha$ -GalCer-induced IFN- $\gamma$  elevation, but not a rise in IL-4 (Fig. 1B). These data clearly show that a single injection of  $\alpha$ -GalCer stimulated secretion of both Th1 and Th2 cytokines, whereas repeated injections induced only Th2 cytokine secretion.

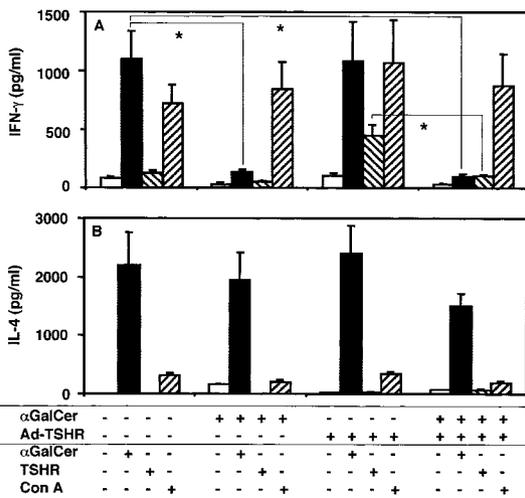
The next experiments were conducted with in vitro cultured splenocytes. In vitro stimulation of splenocytes from naive mice with  $\alpha$ -GalCer or Con A produced both IFN- $\gamma$  and IL-4 (Fig. 2). In contrast, the splenocytes from  $\alpha$ -GalCer-primed mice on days -10, -7, and -3 secreted IL-4, but failed to produce IFN- $\gamma$  in response to in vitro stimulation with  $\alpha$ -GalCer (*p* < 0.001), although in vivo priming with  $\alpha$ -GalCer did not alter cytokine responses to Con A. These data are consistent with the above-mentioned in vivo data, indicating that repeated injection of  $\alpha$ -GalCer selectively suppressed  $\alpha$ -GalCer-induced splenocyte secretion of the Th1 cytokine.



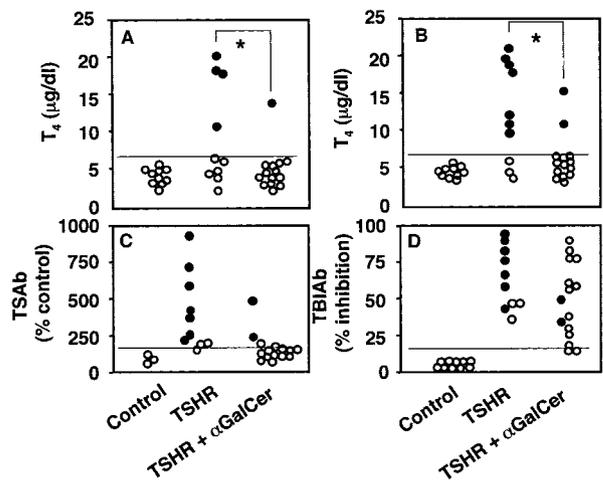
**FIGURE 1.** In vivo production of IFN- $\gamma$  and IL-4 in mice primed with  $\alpha$ -GalCer. Mice pretreated with i.p. injection of vehicle (A) or 100  $\mu$ g/kg  $\alpha$ -GalCer on days -10, -7, and -3 (B) were i.p. injected with 100  $\mu$ g/kg  $\alpha$ -GalCer and were bled at the time points indicated. Serum IFN- $\gamma$  and IL-4 were measured by ELISA. Data are the mean  $\pm$  SE of three mice per group and are expressed as picograms per milliliter.

Cytokine production from splenocytes of mice immunized with Ad-TSHR alone or in combination with  $\alpha$ -GalCer was also evaluated. As we have previously shown (3), splenocytes from mice immunized with Ad-TSHR produced IFN- $\gamma$  in response to in vitro stimulation with TSHR-289 protein. This Ag-specific splenocyte secretion of IFN- $\gamma$  was almost completely suppressed by prior in vivo injection of  $\alpha$ -GalCer (Fig. 2;  $p < 0.001$ ).

We then examined the prophylactic effect of  $\alpha$ -GalCer on Graves' disease induction. Mice were injected with Ad-TSHR alone or in combination with  $\alpha$ -GalCer (100  $\mu$ g/kg) on the day of immunization and 3 and 7 days later in each immunization. Serum  $T_4$  was measured 2 and 8 wk after the last immunization. Elevated  $T_4$  levels (greater than the mean + 3 SD of control mice) were observed in 40% (4 of 10) and 70% (7 of 10) of Ad-TSHR-immunized mice (TSHR group) at these two time points vs 7% (1 of



**FIGURE 2.** In vitro secretion of IFN- $\gamma$  and IL-4 from splenocytes of mice immunized with Ad-TSHR and/or  $\alpha$ -GalCer. Splenocytes were prepared from mice injected with vehicle or 100  $\mu$ g/kg  $\alpha$ -GalCer on days -10, -7, and -3 and/or with Ad-TSHR on day -10 and were stimulated in vitro with 50 ng/ml  $\alpha$ -GalCer, 5  $\mu$ g/ml TSHR-289 protein, or 5  $\mu$ g/ml Con A for 5 days. IFN- $\gamma$  (A) and IL-4 (B) levels in the culture supernatants were measured by ELISA. Data are the means  $\pm$  SD of three mice per group and are expressed as picograms per milliliter. \*, Significant difference by Mann-Whitney  $U$  test.

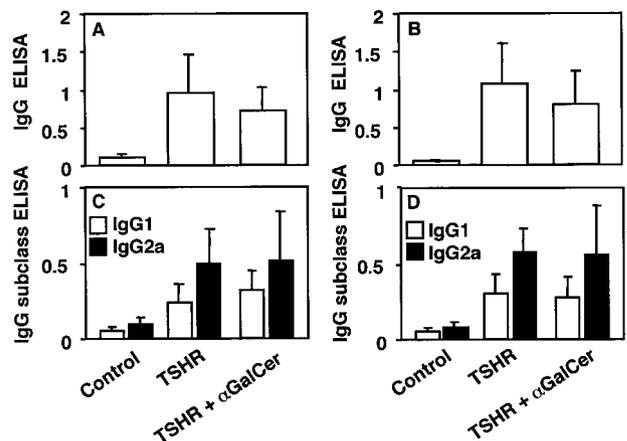


**FIGURE 3.**  $T_4$ , TSAb, and TBIAb values in mice immunized with Ad-TSHR alone or in combination with  $\alpha$ -GalCer in a prophylactic setting.  $T_4$  were determined 2 and 8 wk (A and B), and TSAb and TBIAb were measured 8 wk (C and D) after the third immunization. Data are the means of duplicate determinations.  $\circ$ , Euthyroid mice;  $\bullet$ , hyperthyroid mice; horizontal lines, normal upper limits of each assay; \*, significant difference by  $\chi^2$  test.

15) and 12% (2 of 15) in Ad-TSHR-immunized and  $\alpha$ -GalCer-primed mice (TSHR plus  $\alpha$ -GalCer group;  $p = 0.04$  and  $p < 0.001$ ; Fig. 3, A and B).

Abs against TSHR were measured with mouse sera. IgG and IgG subclass ELISA were performed with sera obtained 2 and 8 wk after the last immunization, and TSAb and TBIAb were measured with sera obtained 8 wk after the last immunization. Most sera from hyperthyroid mice exhibited positive signals in TSAb assay (Fig. 3C), data compatible with a causative role for TSAb in autoimmune hyperthyroidism (1). In contrast, mice in both groups showed similar Ab titers in TBIAb assays and ELISAs (Fig. 3D and Fig. 4, A and B). IgG1 and IgG2a titers were also similar (Fig. 4, C and D). Thus, there is no difference in the ratios of IgG1/IgG2a between the two groups.

The therapeutic efficacy of  $\alpha$ -GalCer was next evaluated. In this experiment,  $\alpha$ -GalCer treatment was initiated 1 wk after the third



**FIGURE 4.** Titers of IgG, IgG1, and IgG2a anti-TSHR Abs determined by ELISA. ELISA for IgG, IgG1, and IgG2a of anti-TSHR Abs were performed with sera obtained 2 wk (A and C) and 8 wk (B and D) after the third immunization. Data are the mean  $\pm$  SD obtained with sera diluted 1/300 ( $n = 10, 10,$  and  $15$  in control, TSHR group, and TSHR plus  $\alpha$ -GalCer group, respectively).

injection of Ad-TSHR when the effector cells were already fully primed.  $\alpha$ -GalCer (300  $\mu$ g/kg) was i.p. administered twice a week for 7 wk because the previous study showing successful treatment of type 1 diabetes with  $\alpha$ -GalCer used the higher dose of  $\alpha$ -GalCer (21). Administration of  $\alpha$ -GalCer proved ineffective in curing Graves' hyperthyroidism; the incidence of disease was essentially the same in the two groups regardless of treatment with  $\alpha$ -GalCer (Fig. 5).

Thus, simultaneous administration of  $\alpha$ -GalCer almost completely suppressed Ag-specific splenocyte secretion of IFN- $\gamma$  and protected Ad-TSHR-immunized mice from Graves' disease, but  $\alpha$ -GalCer had no therapeutic effect.

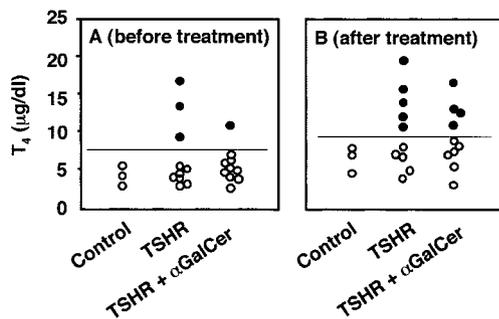
#### Effect of *S. mansoni* infection on Graves' disease induction

We used a low dose of cercariae (15/mouse) to infect BALB/c mice, which allowed long term survival, but led to patent, egg-laying infections in 80–90% of mice. Mice with unsuccessful infection were omitted from the study. Ab responses against SWA and SEA after infection with *S. mansoni* cercariae were determined 3.5 and 7 wk p.i., respectively. Anti-SWA Abs were of IgG1, IgG2a, or both subclasses (Fig. 6A), but anti-SEA Abs were of IgG1 in individual mice (Fig. 6B), indicating mixed Th1 and Th2 Ab responses to SWA and exclusively a Th2 Ab response to SEA.

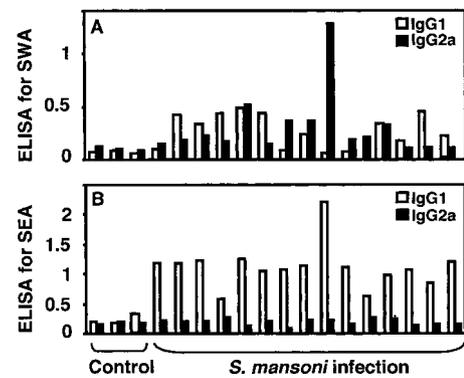
To evaluate the effect of the sustained Th2 immune deviation by *S. mansoni* infection on induction of Graves' disease, mice infected with *S. mansoni* were subsequently injected with Ad-TSHR three times (8 wk p.i.). Elevated  $T_4$  levels were observed in 63% (5 of 8) and 50% (4 of 8) of the TSHR group at the two time points vs 20% (3 of 15) and 7% (1 of 15) in the TSHR plus *S. mansoni* group ( $p = 0.042$  and  $p = 0.016$ , respectively; Fig. 7, A and B).

TSAb was positive in most hyperthyroid mice (Fig. 7C). TBIAb values were similar between the two groups (Fig. 7D). However, TSHR Ab titers determined by ELISA were slightly, but significantly, lower in the TSHR plus *S. mansoni* group than in the TSHR group ( $p = 0.012$  and  $p = 0.022$  at the two time points; Fig. 8, A and B), although there was considerable variation between individual mice. IgG1 levels were similar in the two groups, but IgG2a titers were significantly lower in the TSHR plus *S. mansoni* group than in the TSHR group ( $p = 0.017$  and  $p = 0.016$ ; Fig. 8, C and D). Thus, IgG1/IgG2a ratios were significantly elevated in the TSHR plus *S. mansoni* group ( $p = 0.011$  and  $p = 0.002$ ; Fig. 8, E and F).

In contrast to prior infection with *S. mansoni*, concurrent infection with *S. mansoni* had little effect on disease induction. Thus,



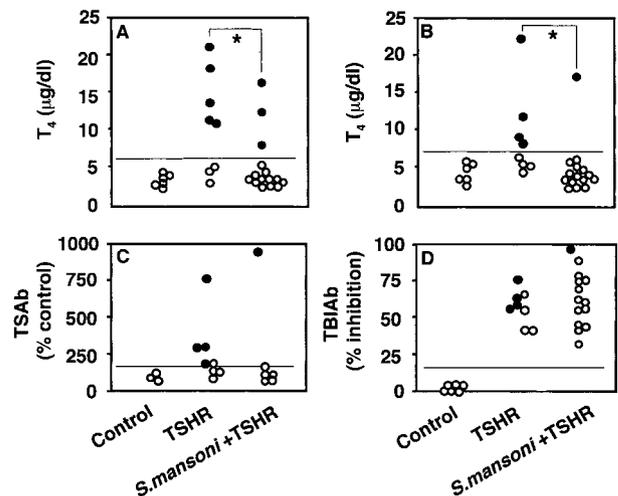
**FIGURE 5.**  $T_4$  levels in mice immunized with Ad-TSHR and treated with vehicle or  $\alpha$ -GalCer in a therapeutic setting. Mice were immunized three times with Ad-TSHR, followed by treatment with vehicle or 300  $\mu$ g/kg  $\alpha$ -GalCer twice a week for 7 wk.  $T_4$  were determined 1 wk (A) and 8 wk (B) after the third immunization with Ad-TSHR. Data are the means of duplicate determinations.  $\circ$ , Euthyroid mice;  $\bullet$ , hyperthyroid mice; horizontal lines, the normal upper limits of the assay.



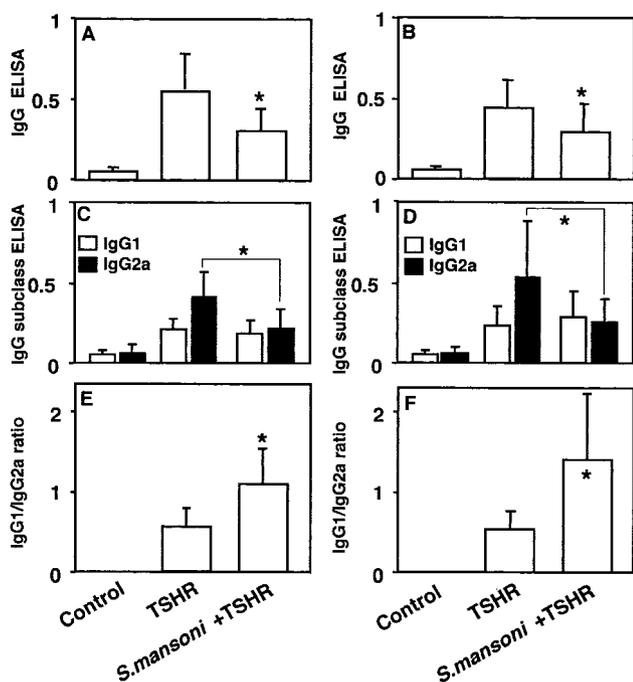
**FIGURE 6.** Titers of anti-SWA and SEA Abs determined by ELISA in mice infected with *S. mansoni* cercariae. ELISA for IgG1 and IgG2a of anti-SWA and SEA Abs were performed with sera obtained 3.5 and 7 wk p.i., respectively. Data are the means of duplicate determinations with 100-fold diluted sera.

the incidence of disease was similar in the TSHR and TSHR plus *S. mansoni* groups at the two time points (Fig. 9, A and B). Ab titers measured by TBIAb and ELISA were also similar in the two groups 8 wk after the last immunization (Fig. 9, C and D). Of interest, the ratios of IgG1/IgG2a anti-TSHR Abs were essentially same in the two groups 2 wk after the third injection of Ad-TSHR, but were higher in the TSHR plus *S. mansoni* group than in the TSHR group 8 wk after the last immunization ( $p = 0.048$ ) due to the decreased IgG2a titers ( $p = 0.002$ ; Fig. 9, E–H).

Splenocyte secretions of Th1 and Th2 cytokines, IFN- $\gamma$ , IL-4, and IL-10, were also studied (Fig. 10). Splenocytes from mice immunized with Ad-TSHR after *S. mansoni* infection exhibited elevated basal levels of IFN- $\gamma$  ( $p = 0.006$ ) and impaired production of IFN- $\gamma$  in response to in vitro stimulation with TSHR-289 (Fig. 10A). These data are reminiscent of our previous data obtained with Ad-IL-4 (3). Splenocytes from Ad-TSHR-immunized mice also produced IL-10 in response to TSHR-289, and prior infection with *S. mansoni* increased not only the basal, but also



**FIGURE 7.**  $T_4$ , TSAb, and TBIAb values in mice immunized with Ad-TSHR and/or infected with *S. mansoni*.  $T_4$  were determined 2 and 8 wk (A and B), and TSAb and TBIAb were measured 8 wk (C and D) after the third immunization. Data are the means of duplicate determinations in individual mice.  $\circ$ , Hyperthyroid mice;  $\bullet$ , euthyroid mice; horizontal lines, the normal upper limits of each assay; \*, significant difference by  $\chi^2$  test.



**FIGURE 8.** Titers of IgG, IgG1, and IgG2a anti-TSHR Abs determined by ELISA. ELISAs for IgG, IgG1, and IgG2a of anti-TSHR Abs were performed with sera obtained 2 wk (A, C, and E) and 8 wk (B, D, and F) after the third immunization. Data are the mean  $\pm$  SD obtained with 300-fold diluted sera ( $n = 6, 8,$  and  $15$  in control, TSHR, and TSHR plus *S. mansoni* groups). \*, Significant difference by Mann-Whitney *U* test.

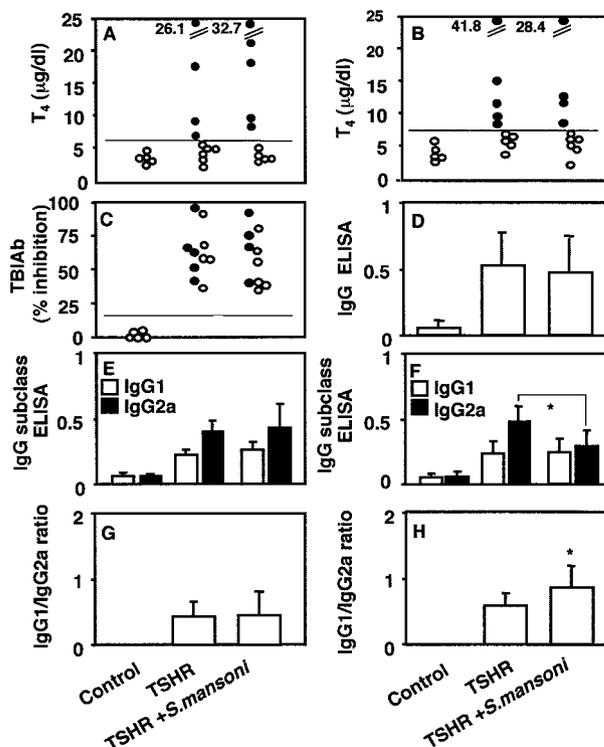
Ag-induced, levels of IL-10 ( $p = 0.004$  and  $p = 0.007$ ), suggesting no effect on the responsiveness of IL-10 to TSHR-289 stimulation (Fig. 10B). In mice concurrently infected with Ad-TSHR and *S. mansoni*, splenocyte secretion of IFN- $\gamma$  was intact (Fig. 10C). Thus, the Th2-deviating immune response is associated with the blunt IFN- $\gamma$  response of splenocytes to stimulation with TSHR Ag. IL-4 was below detectable levels for all condition (data not shown).

To confirm the different effects of *S. mansoni* eggs and worms, mice were injected with Ad-TSHR in combination with SWA or SEA proteins on days  $-7, -4,$  and  $0$  of each immunization. Injection of SWA and SEA elicited mixed Th1 and Th2 Ab responses and a Th2 Ab response, respectively (Fig. 11A). The incidence of Graves' hyperthyroidism was significantly lower in the TSHR plus SEA group than in the TSHR plus SWA group 8 wk after the last immunization ( $p = 0.02$ ; 20% (2 of 10) vs 70% (7 of 10); Fig. 11B).

Thus, prior infection with *S. mansoni* suppressed Graves' hyperthyroidism, IgG2a anti-TSHR Ab titers, and Ag-specific splenocyte secretion of IFN- $\gamma$ . This protective effect is probably mediated by Ags enriched in soluble egg extracts.

## Discussion

In this article we studied the effect of sustained Th2 immune deviations induced by *S. mansoni* infection and  $\alpha$ -GalCer on Graves' disease using a mouse Graves' model we have recently established (2). Our results clearly demonstrate that the Th2-dominant immune response against *S. mansoni* infection and preferential secretion of the Th2 cytokine by repetitive injection of  $\alpha$ -GalCer induced deviation of the anti-TSHR immune response away from Th1. This immune deviation is due to suppression of the Th1 immune response rather than enhancement of the Th2 immune response because prior infection with *S. mansoni* and  $\alpha$ -GalCer suppressed

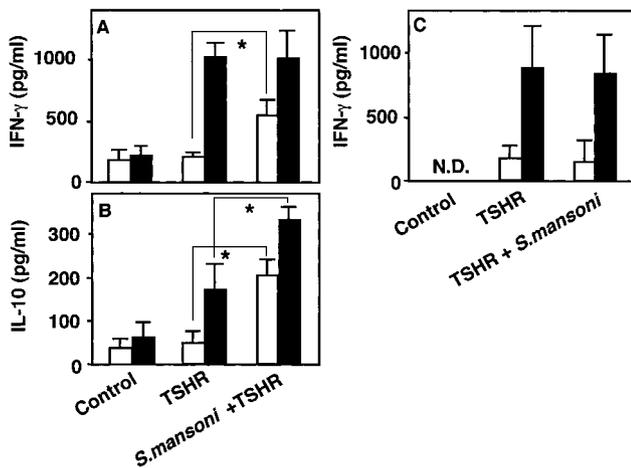


**FIGURE 9.**  $T_4$  and titers of IgG1 and IgG2a anti-TSHR Abs determined by ELISA in mice infected with TSHR alone or simultaneously infected with TSHR and *S. mansoni*. These values were determined 2 wk (A and E) and 8 wk (B–D and F) after the third immunization. Data are the means of duplicate assays (A–C) or the mean  $\pm$  SD with 300-fold diluted sera (D–F).  $\circ$ , Euthyroid mice;  $\bullet$ , hyperthyroid mice; horizontal lines, the normal upper limits of each assay; \*, significant difference by Mann-Whitney *U* test.

Ag-specific splenocyte secretion of IFN- $\gamma$  and/or lowered IgG2a-type TSHR Ab titers. The effect of  $\alpha$ -GalCer on promotion of the Ag-specific Th2 response is controversial; several reports did (20, 21, 25–27), but the others did not (18, 45), find differentiation of Ag-specific T cells into Th2 cells by  $\alpha$ -GalCer. In contrast, *S. mansoni* eggs constantly enhance the Th2 immune response to nonparasite Ags (33, 34, 46, 47). The reason(s) for the failure to induce Th2 response to TSHR by *S. mansoni* is unclear.

We then showed that this immune deviation at the time of Ag priming, not after activation of the TSHR-specific immune response, prevented disease development. Similar data have been obtained in our recent study (3), showing prevention of Graves' hyperthyroidism by transient expression of IL-4 by adenovirus at the time of Ag presentation. Thus, suppression of the Ag-specific Th1 immune response appears to be crucial to prevent the development of a pathogenic anti-TSHR immune response, but may not be able to revert a fully activated anti-TSHR immune response. This conclusion is consistent with 1) a report by Beaudoin et al. (45) that NKT cells can protect against diabetes induced by passive transfer of naive pathogenic T cells, but cannot prevent diabetes when the pathogenic T cells are fully differentiated into Th1 cells before transfer, in NOD mice; and 2) a report by Boitelle et al. (48) showing suppression of Ag-specific T cell proliferation during the early stage of the primary immune response by parasite infection.

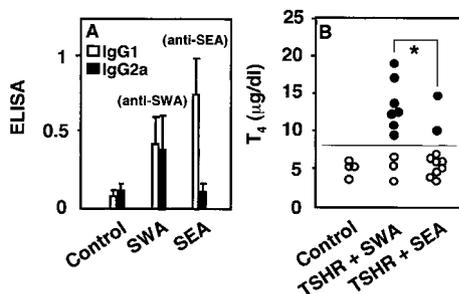
Recent studies show that induction of tolerogenic APCs (22, 49, 50) or regulatory T cells/regulatory cytokines (18, 33, 51, 52) also participates in *S. mansoni*- and  $\alpha$ -GalCer-mediated amelioration of autoimmune diseases. Furthermore, of interest, Zaccone et al. (38) have recently demonstrated that treatment with SWA and SEA of



**FIGURE 10.** In vitro secretion of IFN- $\gamma$  and IL-10 from splenocytes of mice infected with Ad-TSHR and/or *S. mansoni*. Splenocytes were prepared from mice infected with *S. mansoni*, followed by injection of Ad-TSHR 8 wk p.i. (A and B) or from mice simultaneously infected with Ad-TSHR and *S. mansoni* (C) and were stimulated with 5  $\mu$ g/ml TSHR-289 protein for 5 days. IFN- $\gamma$  (A and C) and IL-10 (B) levels in the culture supernatants were measured by ELISA. Data are the mean  $\pm$  SD of three mice per group and are expressed as picograms per milliliter.  $\square$ , Splenocytes cultured in the absence of TSHR-289;  $\blacksquare$ , splenocytes cultured in the presence of TSHR-289; \*, significant difference by Mann-Whitney *U* test.

*S. mansoni* increased the number of NKT cells in NOD mice. As *S. mansoni* eggs and worms express a variety of glycoconjugates, including galactosylceramide as mentioned in the introduction (36), the mechanisms for the protective effects of *S. mansoni* and  $\alpha$ -GalCer may at least partly overlap each other. However, the different patterns of suppression of Ag-specific splenocyte secretion of IFN- $\gamma$  (Fig. 2 vs Fig. 10) and the different effects on IgG2a Ab titers (Fig. 4 vs Fig. 8) by  $\alpha$ -GalCer and *S. mansoni* suggest that the effects are not entirely identical. In this regard, it may be worth noting that Ad-IL-4 (3) and *S. mansoni* both induce partial suppression of Ag-specific splenocyte secretion of IFN- $\gamma$  and elevation of IgG1/IgG2a ratios of TSHR Abs. Thus, the effects of transiently overexpressed exogenous IL-4 by Ad-IL-4 (3) and more physiological, sustained Th2 immune deviation by *S. mansoni* are very similar.

Although the titers of anti-TSHR Abs determined by TBIAb assays and ELISAs were largely unaffected, generation of TSAb, i.e., Abs recognizing a particular epitope(s) necessary for TSHR activation, was selectively suppressed. In other words, the immune



**FIGURE 11.** Titers of anti-SWA and SEA Abs and  $T_4$  values in mice immunized with Ad-TSHR in combination with SWA or SEA. These values were determined with sera obtained 8 wk after the third immunization. Data are the mean  $\pm$  SD with 100-fold diluted sera (A) or the mean of duplicate assays (B).  $\circ$ , Euthyroid mice;  $\bullet$ , hyperthyroid mice; horizontal line, normal upper limit of  $T_4$  assay; \*, significant difference by  $\chi^2$  test.

response to TSHR was still occurring, but development of a pathogenic anti-TSHR response was prevented. We have recently found similar data with Ad-IL-4 (3). These findings are consistent with the previous reports that lymphocyte infiltration of pancreas and CNS could not be completely suppressed by  $\alpha$ -GalCer treatment, NKT cell overexpression, or *S. mansoni* infection in diabetes and EAE models, albeit less severe (18, 20, 23, 37). In diabetes, lymphocytic infiltration into the pancreas still occurs, but remains largely peri-islet, which is therefore not pathogenic and is unable to mediate  $\beta$  cell destruction.

The present study shows not only splenocyte secretion of IL-10 in response to in vitro stimulation with TSHR Ag, but also elevation of the basal levels of IL-10 by *S. mansoni* infection. These data may be of interest in terms of the dual functions of IL-10, namely, pro- and anti-inflammatory actions (53). IL-10 is reported to suppress Th1 autoimmune diseases (54, 55) and also to aggravate autoantibody-mediated autoimmune diseases such as lupus and myasthenia gravis (56, 57). IL-10 is reported to be involved in parasite-mediated amelioration of autoimmune diseases; SEA decreased IL-12 production and increased IL-10 secretion from dendritic cells in the presence of LPS (38). These data suggest that IL-10 administration may produce either desirable consequence in Graves' model, i.e., reduction of Ab production and suppression of disease, or undesirable effect, i.e., exacerbation of disease. Further study will be necessary to address this issue.

It may be worth noting that the number of NKT cells in Graves' patients is reported to be normal (16), although a causative link between functional defect of NKT cells and development of Graves' disease is at present unknown in humans. In this regard, it is intriguing that the suppressive effect of  $\alpha$ -GalCer on Graves' disease is observed in BALB/c mice that have numerically and functionally normal NKT cells (58). Our data suggest that NKT cells may play a crucial role in controlling the pathogenic anti-TSHR immune response in Graves' disease.

In conclusion, prior infection with *S. mansoni* and  $\alpha$ -GalCer prevent Graves' hyperthyroidism in our mouse model, presumably by inhibiting activation of a Th1-type anti-TSHR immune response at the time of Ag priming. These data reinforce the significance of the Th1 immune response in the pathogenesis of Graves' disease that we have previously proposed (3). Although our animal model may not perfectly mimic Graves' disease in humans, data supporting the involvement of the Th1 immune response in Graves' patients, for example, Th1-type TSAbs (59), are indeed present.

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