

Prevention of Autoantibody-Mediated Graves'-Like Hyperthyroidism in Mice with IL-4, a Th2 Cytokine

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Graves' hyperthyroidism has long been considered to be a Th2-type autoimmune disease because it is directly mediated by autoantibodies against the thyrotropin receptor (TSHR). However, several lines of evidence have recently challenged this concept. The present study evaluated the Th1/Th2 paradigm in Graves' disease using a recently established murine model involving injection of adenovirus expressing the TSHR (AdCMVTSHR). Coinjection with adenovirus expressing IL-4 (AdRGDCMVIL-4) decreased the ratio of Th1/Th2-type anti-TSHR Ab subclasses (IgG2a/IgG1) and suppressed the production of IFN- γ by splenocytes in response to TSHR Ag. Importantly, immune deviation toward Th2 was accompanied by significant inhibition of thyroid-stimulating Ab production and reduction in hyperthyroidism. However, in a therapeutic setting, injection of AdRGDCMVIL-4 alone or in combination with AdCMVTSHR into hyperthyroid mice had no beneficial effect. In contrast, coinjection of adenoviruses expressing IL-12 and the TSHR promoted the differentiation of Th1-type anti-TSHR immune responses as demonstrated by augmented Ag-specific IFN- γ secretion from splenocytes without changing disease incidence. Coinjection of adenoviral vectors expressing IL-4 or IL-12 had no effect on the titers of anti-TSHR Abs determined by ELISA or thyroid-stimulating hormone-binding inhibiting Ig assays, suggesting that Ab quality, not quantity, is responsible for disease induction. Our observations demonstrate the critical role of Th1 immune responses in a murine model of Graves' hyperthyroidism. These data may raise a cautionary note for therapeutic strategies aimed at reversing Th2-mediated autoimmune responses in Graves' disease in humans. *The Journal of Immunology*, 2003, 170: 3522–3527.

Graves' disease is an Ab-mediated organ-specific autoimmune disease in which stimulating anti-thyrotropin receptor (TSHR)² autoantibodies (thyroid-stimulating Abs, TSAb) are responsible for hyperthyroidism and goiter by overstimulating the TSHR (reviewed in Refs. 1 and 2). Because of the role of autoantibodies, Graves' disease has long been considered to be a Th2-dominated disease. Support for this hypothesis includes features of atopy such as elevated serum IgE in some Graves' patients (3) as well as the induction of Graves' disease in patients with multiple sclerosis (a Th1-dominated autoimmune disease) following treatment with monoclonal anti-CD52 Ab (4). On the other hand, cytokine expression profiles in sera and thyroid tissues from Graves' patients indicate a mixed Th1/Th2 immune response (reviewed in Ref. 5). Moreover, Graves' disease generally ameliorates during pregnancy, a Th2-dominant state (6), and even more important, TSAb are IgG1 (7), a Th1-type subclass in humans.

Th1/Th2-type responses analyzed using two murine models of Graves' disease (8, 9) provide conflicting observations (10–14).

The pioneering "Shimojo" model involves injecting fibroblasts co-expressing the human TSHR and MHC class II into syngeneic AKR/N mice (8). In this model, a Th1 adjuvant (CFA) delays and a Th2 adjuvant (alum) accelerates disease induction, suggesting the importance of Th2 immune responses (10). However, injection of the fibroblasts is associated with marked splenocyte production of the Th1 cytokine IFN- γ but not the Th2 cytokine IL-4 (11). In outbred NMRI mice vaccinated with TSHR-DNA, the presence of B cells, IL-4-producing T cells, and mast cells infiltrating the thyroid glands reflects Th2 responses (9). On the other hand, the same researchers have shown that anti-TSHR mAbs established from the spleens of TSHR DNA-vaccinated BALB/c mice are all IgG2a (12), a murine Th1 subclass. Consistent with the latter observation, splenocytes from TSHR-DNA-vaccinated BALB/c mice secrete IFN- γ , but not IL-4, in response to TSHR Ag (13) and T cell responses to TSHR-DNA vaccination are generated in IFN- γ KO BALB/c mice (14). The above discrepancies could be related to studies being performed in different laboratories, with different mouse strains and different cell types. Moreover, neither the Shimojo model nor naked DNA vaccination are suitable for definitive testing of the Th1/Th2 paradigm in Graves' disease for the following reasons: 1) nonspecific immune activation induced by costimulatory molecules expressed on the injected fibroblasts prevents detailed Ab analysis or in vitro studies in the Shimojo model (11) and 2) induction of TSAb is very rare and hyperthyroidism fails to develop in inbred mice vaccinated with TSHR-DNA (13).

A novel murine model developed in our laboratory provides the opportunity for in vivo and in vitro analysis of the Th1/Th2 paradigm in Graves' disease. Intramuscular injection of adenovirus coding the TSHR induces TSAb and hyperthyroidism in >50% of female BALB/c mice (15). In the present study, we determined the outcome of injecting adenoviruses expressing the TSHR in combination with adenovirus expressing IL-4 or IL-12. We found that

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² Abbreviations used in this paper: TSHR, thyrotropin receptor; TSAb, thyroid-stimulating Ab; TSH, thyroid-stimulating hormone; TBIAb, TSH-binding inhibiting Ab; TBAb, TSH-blocking Ab; T₄, thyroxine; MOI, multiplicity of infection; AIHA, autoimmune hemolytic anemia.

coexpression of IL-4 significantly skewed the Ag-specific immune response to Th2 and inhibited the induction of Graves' hyperthyroidism. In contrast, immune deviation to Th1 by coexpression of IL-12 had no effect on disease induction. Our findings reinforce the implication of the TSAbs subclass data in humans, namely, that Th1-type responses mediate Graves' disease.

Materials and Methods

Adenovirus vectors used

Adenovirus expressing the human TSHR (AdCMVTSHR) has previously been described (15). Adenoviruses expressing mouse IL-12 or mouse IL-4 were constructed according to a recently described method (16, 17). The plasmid pBSKS⁺ IL-12BIA containing cDNAs for mouse IL-12 p35 and p40 linked with the internal ribosome entry site gene of encephalomyocarditis virus (18) (kindly provided by Dr. H. Yamamoto, Osaka University, Osaka, Japan) was digested with *EcoRV* and *NotI*. The plasmid pBSKS⁺ mIL4 containing cDNA for mouse IL-4 (RIKEN DNA Bank, Tsukuba, Japan) was digested with *EcoRI*, blunt-ended with DNA T₄ polymerase, and digested with *NotI*. These fragments were ligated into pHMCMV6 (16) which had been digested with *NheI*, blunt-ended, and digested with *XbaI*. The resultant plasmids pHMCMVIL-12 and pHMCMVIL-4 were then digested with *I-CeuI*/PI-*SceI*, and ligated into *I-CeuI*/PI-*SceI*-digested pAdHM15-RGD (17). pAdHMRGDCMVIL-12 and pAdHMRGDCMVIL-4 were linearized with *PacI* and transfected into 293 human embryonal kidney cells with PolyFect (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Recombinant adenoviruses expressing IL-12 or IL-4 (designated AdRGDCMVIL-12 and AdRGDCMVIL-4) were then plaque purified. Adenovirus was propagated in 293 human embryonal kidney cells and purified through two rounds of CsCl density gradient centrifugation. The viral particle concentration was determined as previously described (15). The multiplicity of infection (MOI) was defined as the ratio of total number of particles used in a particular infection divided by the number of cells.

Immunization protocol

Female BALB/c mice (6 wk old) were purchased from Charles River Breeding Laboratory (Tokyo, Japan). All experiments were conducted in accordance with the principles and procedures outlined in the *Guidelines for the Care and Use of Laboratory Animals* at the Nagasaki University (Nagasaki, Japan). Mice were kept in a specific pathogen-free condition through the experiments. For immunization, mice were injected i.m. with 50 μ l of PBS containing 10¹¹ particles of AdCMVTSHR alone or in combination with 5 $\times$ 10¹⁰ particles of AdRGDCMVIL-4 or AdRGDCMVIL-12. The same immunization schedule was repeated twice at 3-wk intervals. Blood was drawn from tails before the second and third immunizations and 3 wk after the third immunization and by cardiac puncture 8 wk after the third immunization. Thyroid glands were preserved for histology.

In a therapeutic setting, AdRGDCMVIL-4 (5 $\times$ 10¹⁰ particles/mouse) alone or in combination with AdCMVTSHR (10¹¹ particles/mouse) was injected twice at a 2-wk interval into mice that were in a hyperthyroid state 8 wk after three injections of AdCMVTSHR.

Thyroxine (T₄), TSAbs, thyroid-stimulating hormone (TSH)-blocking Abs (TBAbs), and TSH-binding inhibiting Ab (TBIAb) measurements

Total T₄ in murine sera was measured with a commercially available RIA kit (RIA-gnost tT₄; Nippon Schering, Osaka, Japan). The normal range was defined as the mean $\pm$ 3 SD of control mice.

TSAbs activities in murine sera were measured with FRTL5 cells as previously described (15). The cells were seeded at 3 $\times$ 10⁴ cells/well in a 96-well culture plate and incubated in 50 μ l of hypotonic HBSS containing 0.5 mM isobutyl-methylxanthine, 20 mM HEPES, 0.25% BSA, and 5 μ 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 over 150% of control mice was judged as positive. TBAbs activities were measured in the same hypotonic buffer supplemented with 100 mU/ml bovine TSH (Sigma-Aldrich, St. Louis, MO) and were expressed as percent inhibition of TSH-induced cAMP generation by test sera.

TBIAb values were determined with a commercially available TRAb kit (BRAHMS Diagnostica, Berlin, Germany). Ten microliters of serum was used for each assay. A value over 15% inhibition of control binding was judged as positive.

ELISA for TSHR Abs

ELISA for detecting mouse IgG Abs against the TSHR was as previously reported (11, 13) with minor modifications. TSHR-289, a variant of the receptor expressed in eukaryotic cells that corresponds approximately to the extracellular A subunit (19), was affinity purified from culture medium (20). ELISA wells were coated overnight with 100 μ l of TSHR-289 protein (1 μ g/ml) and incubated with mouse sera (1/300 dilution). After incubation with HRP-conjugated anti-mouse IgG (diluted 3000 times, A3673; Sigma-Aldrich) or subclass-specific anti-mouse IgG1 and IgG2a (diluted 1000 and 1500 times, respectively; X56 and R19-15; BD Pharmingen, San Diego, CA), color was developed using *o*-phenylenediamine and H₂O₂ as substrate and the OD read at 492 nm.

Cytokine assays

COS cells seeded at 1 $\times$ 10⁵ cells/well in a 24-well culture plate were infected with adenovirus encoding IL-12 or IL-4 at the indicated MOIs. The next day, the cells were washed three times with PBS and the culture was continued in fresh medium for 2 days. The concentrations of IL-12 or IL-4 in culture supernatants were determined with ELISA kits (BioSource International, Camarillo, CA) according to the manufacturer's protocols. Serum levels of IL-4 and IL-12 were also measured with ELISA kits (BioSource International).

Splenocytes were cultured (triplicate aliquots) at 4 $\times$ 10⁵ cells/well in a 96-well round-bottom plate in the presence or absence of TSHR-289 protein (5 μ g/ml). Five days later, the concentrations of IFN- γ and IL-4 in the medium were determined with ELISA kits (BioSource International). Cytokine production was expressed as picograms per milliliter using standard curves of recombinant murine IL-12 and IL-4 or as a fold increase relative to cultures without TSHR Ag.

Thyroid histology

Thyroid tissues were removed and fixed with 10% Formalin in PBS. Tissues were embedded in paraffin and 5- μ m thick sections were prepared and stained with H&E.

Results

To investigate the effect of coexpression of IL4 or IL-12 in our murine Graves' model (15), we constructed replication-defective recombinant adenoviruses encoding IL-4 and IL-12 (AdRGDCMVIL-4 and AdRGDCMVIL-12). These adenoviruses have a RGD sequence, the binding site for $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -integrin, on the HI loop of the fiber knob, thus facilitating RGD/integrin-dependent viral infection (17). Although this mutant virus retains the binding activity with its cognate receptor, it exhibits higher infectivity to a variety of cells than wild-type adenovirus (17). COS cells infected

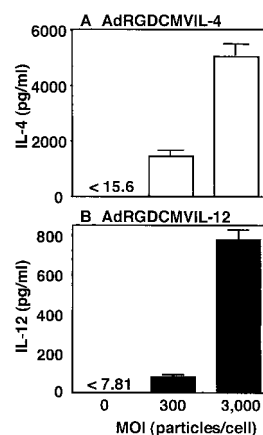


FIGURE 1. In vitro production of cytokines in COS cells infected with adenoviruses expressing either IL-4 or IL-12. The concentrations of IL-4 and IL-12 released for 2 days from COS cells infected with AdRGDCMVIL-4 or AdRGDCMVIL-12, respectively, at the MOIs indicated were measured as described in *Materials and Methods*. Data are means $\pm$ SD ($n = 3$) in one of two independent experiments.

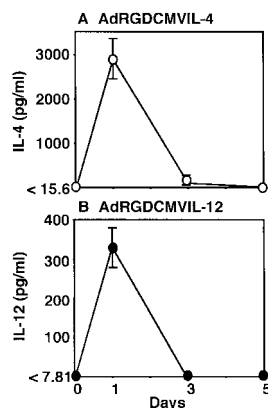


FIGURE 2. In vivo production of cytokines in mice injected with adenoviruses expressing either IL-4 or IL-12. Mice were injected i.m. with AdRGDCMVIL-4 or AdRGDCMVIL-12 (5×10^{10} particles/mouse; day 0) and bled on days 1, 3, and 5 for cytokine assays. Data are means $\pm$ SD of three mice in each group.

with AdRGDCMVIL-4 and AdRGDCMVIL-12 produced significant amounts of IL-4 and IL-12, respectively, in a MOI-dependent manner (Fig. 1). Furthermore, i.m. injection of AdRGDCMVIL-4 and AdRGDCMVIL-12 (5×10^{10} particles/mouse) to mice also increased serum concentrations of IL-4 and IL-12, respectively, although the expression was transient (Fig. 2). In vitro infection (MOI of 3000 particles/cell) or in vivo injection (10^{11} particles/

mouse) of AdCMVTSHR did not induce detectable IL-4 or IL-12 (data not shown).

AdCMVTSHR alone (10^{11} particles/mouse) or in combination with AdRGDCMVIL-4 or AdRGDCMVIL-12 (5×10^{10} particles/mouse) was injected into quadriceps of female BALB/c mice three times at 3-wk intervals. As shown in Fig. 3A, some mice in all groups developed hyperthyroidism after the second immunization, and thereafter the incidence of hyperthyroidism increased. In our preliminary experiments, the incidence of hyperthyroidism plateaued 8 wk after the third immunization (data not shown). The serum concentration of T_4 in naive mice was 4.52 ± 0.83 $\mu\text{g/dl}$ (mean $\pm$ SD). On this basis, the normal range of T_4 was defined as 2.03–7.01 $\mu\text{g/dl}$ (mean $\pm$ 3 SD). Disease incidence at this time point was 60% (12 of 20) in mice immunized with AdCMVTSHR alone (TSHR group), similar to our previous study (15, 21), 53% (8 of 15) in those with AdCMVTSHR and AdRGDCMVIL-12 (TSHR plus IL-12), but only 13% (2 of 15) in those with AdCMVTSHR and AdRGDCMVIL-4 (TSHR plus IL-4) (Figs. 3A and 4A). Thus, coexpression of IL-4 significantly suppressed disease induction compared with induction by TSHR alone or by TSHR plus IL-12 ($p = 0.005$ and 0.020 , respectively, χ^2 test).

By ELISA, anti-TSHR of the IgG class were detected 3 wk after the first immunization in all groups of immunized mice and the titers plateaued after the second immunization (Fig. 3B). TBIAb activities were detectable later, after the second immunization (Fig. 3D). It is at present unclear whether the TBIAb assay is less

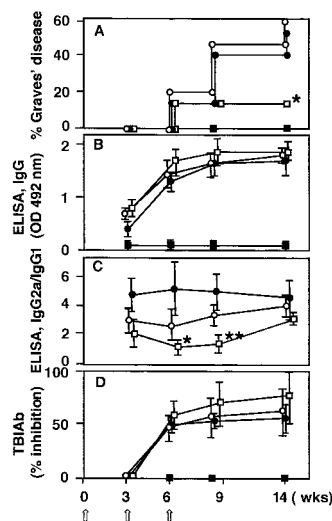


FIGURE 3. Incidence of hyperthyroidism, anti-TSHR Abs in ELISA (IgG, IgG1, and IgG2a), and TBIAb activities in mice immunized with AdCMVTSHR alone or in combination with AdRGDCMVIL-4 or AdRGDCMVIL-12. Groups of mice were immunized with AdCMVTSHR (1×10^{11} particles/mouse) alone or in combination with AdRGDCMVIL-4 or AdRGDCMVIL-12 (5×10^{10} particles/mouse) and bled for T_4 and autoantibody assays just before the second and third immunizations and 3 and 8 wk after the third immunization. A, Mice with T_4 more than mean $\pm$ 3 SD of control mice were judged as hyperthyroid. *, $p = 0.005$ and 0.02 for the TSHR plus IL-4 group vs the TSHR and the TSHR plus IL-12 groups, respectively (χ^2 test). B–D, Anti-TSHR Abs of IgG, IgG1, and IgG2a and TBIAb activities in mouse sera were measured as described in *Materials and Methods*. $\circ$, Mice immunized with AdCMVTSHR alone; $\bullet$, those with AdCMVTSHR and AdRGDCMVIL-12; $\square$, those with AdCMVTSHR and AdRGDCMVIL-4; $\blacksquare$, naive mice. Data are means $\pm$ SD in B and D and means $\pm$ SE in C, each determined in duplicates. Arrows indicate adenovirus injections. * and **, $p = 0.021$ and 0.009 for the TSHR plus IL-4 group vs the TSHR group, respectively (Student's t test).

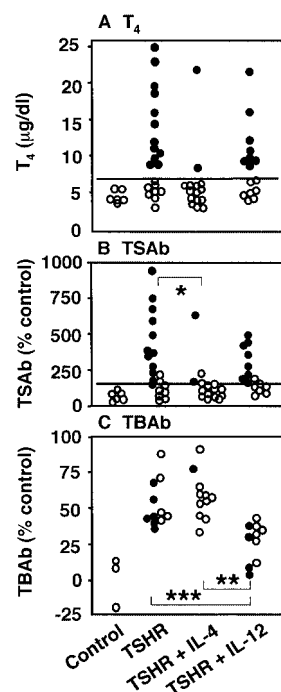


FIGURE 4. T_4 and TSAb and TBAb activities in mice immunized with AdCMVTSHR alone or in combination with AdRGDCMVIL-4 or AdRGDCMVIL-12. T_4 , TSAb, and TBAb activities were determined 8 wk after the third immunization as described in *Materials and Methods*. Data are means of duplicate assays. $\circ$ and $\bullet$, Euthyroid and hyperthyroid mice, respectively. TBAb activities were determined only in mice for which sufficient sera were available ($n = 10$ – 11 in each group). Horizontal lines indicate the normal upper limits of T_4 and TSAb assays; the normal upper limit of TBAb was not calculated because only a small number of control sera ($n = 3$) were available in this assay. B, *, $p = 0.036$ for the TSHR group vs the TSHR plus IL-4 group; C, * and **, $p = 0.0002$ and 0.001 for the TSHR plus IL-4 group vs the TSHR plus IL-4 and TSHR plus IL-12 groups, respectively (Student's t test).

sensitive than ELISA or whether neutral Abs (without TSAb or TBIAb activities) appear first, followed by TBIAb. In contrast to thyroid function mentioned above, there were no differences in the Ab levels measured by ELISA or TBIAb between the mice injected with the different combinations of adenovirus, namely, TSHR, TSHR plus IL-4, or TSHR plus IL-12, during the course of the experiment (Fig. 3, B and D). TSAb and TBAb activities were measured only at the end of the experiments because of limited amounts of sera. TSAb activities, positive in most hyperthyroid mice, were significantly lower in the TSHR plus IL-4 group than in the TSHR group ($p = 0.036$, Student's *t* test; Fig. 4B). TBAb activities were significantly lower in the TSHR plus IL-12 group ($24.3 \pm 13.1\%$, mean $\pm$ SD) than in the TSHR group or the TSHR plus IL-4 group ($51.9 \pm 14.9\%$ and $57.9 \pm 14.9\%$, $p = 0.0002$ and 0.0001 , respectively, Student's *t* test; Fig. 4C).

Th1 vs Th2 balance was examined in two ways: 1) in terms of the IgG subclass-specific ELISA (Fig. 3C) and 2) cytokines secreted by splenocytes in response to TSHR Ag (Fig. 5). The IgG2a:IgG1 (Th1:Th2) ratio of anti-TSHR Abs was significantly lower in the TSHR plus IL-4 group 3 wk after the second and third immunizations as compared with TSHR and TSHR plus IL-12 groups ($p = 0.021$ and 0.009 , respectively, Student's *t* test). However, this difference was not observed before the second immunization or 8 wk after the third immunization ($p = 0.173$ and 0.065). Although the mean IgG2a:IgG1 ratios in the TSHR/IL-12 group were slightly elevated compared with the mean values in the TSHR group throughout the experiment, the differences were not statistically significant.

Splenocytes obtained 10 days after a single injection with AdCMVTSHR secreted IFN- γ in response to TSHR Ag (Fig. 5, A and B). When expressed as fold increase, IFN- γ secretion was significantly lower in the TSHR plus IL-4 group vs the TSHR group

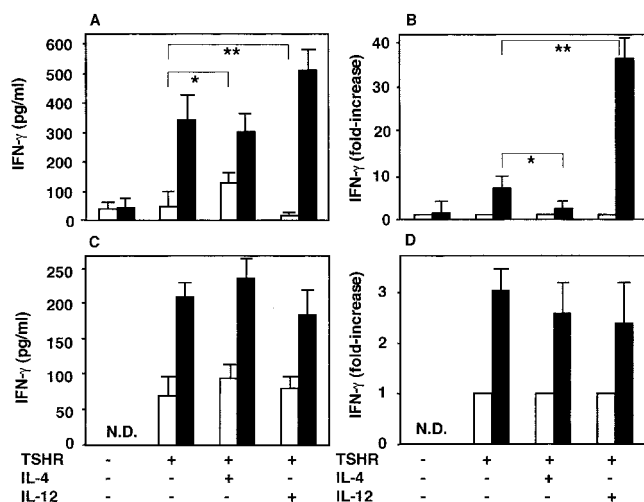


FIGURE 5. IFN- γ production from splenocytes of mice immunized with AdCMVTSHR alone or in combination with AdRGDCMVIL-4 or AdRGDCMVIL-12. Splenocytes were isolated 10 days after a single adenovirus injection (A and B) or 8 wk after three injections (C and D). IFN- γ in culture supernatants of splenocytes incubated in the presence or absence of TSHR Ag (TSHR-289, $5 \mu\text{g/ml}$) for 5 days was determined by ELISA. Data are means $\pm$ SD ($n = 4$) and are expressed either as picograms per milliliter in A and C or as fold increase relative to cultures without TSHR-289 in B and D. $\square$ and $\blacksquare$, Splenocytes cultured in the absence or presence of TSHR-289, respectively. A, *, $p = 0.012$ for the TSHR group vs the TSHR plus IL-4 group; **, $p = 0.027$ for the TSHR group vs the TSHR plus IL-12 group. B, *, $p = 0.011$ for the TSHR group vs the TSHR plus IL-4 group; **, $p = 0.0038$ for the TSHR group vs the TSHR plus IL-12 group (Student's *t* test). ND, Not done.

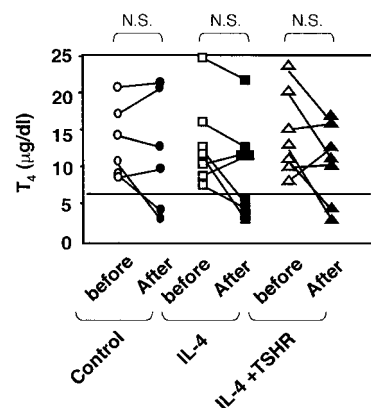


FIGURE 6. T_4 levels in hyperthyroid mice treated with AdRGDCMVIL-4 alone or in combination with AdCMVTSHR. Groups of mice ($n = 6-7$) that were in a hyperthyroid state 8 wk after three injections of AdCMVTSHR were treated with two i.m. injections of AdRGDCMVIL-4 (5×10^{10} particles/mouse) alone or in combination with AdCMVTSHR (10^{11} particles/mouse) at a 2-wk interval. Serum T_4 levels were measured before ($\blacksquare$) and 2 wk after the second injection ($\square$).

(~6-fold vs ~2.5-fold, $p = 0.011$) and significantly higher in the TSHR plus IL-12 group as compared with the TSHR group (~36-fold, $p = 0.0038$; Fig. 5B). However, this difference was no longer observed in splenocytes isolated from mice at the end of the experiments (Fig. 5, C and D). These data clearly demonstrate the shifting of immune response, albeit transiently, to Th1 and Th2 by IL-12 and IL-4, respectively. No IL-4 secretion was observed in mice of any group (data not shown).

Incidentally, the histology of the thyroid glands from hyperthyroid mice was essentially same among the three groups. In particular, the hyperthyroid mice had diffuse goiters with hypertrophy and hypercellularity of thyroid epithelial cells as previously reported (15), findings consistent with those in overstimulated thyroid glands. No lymphocytic infiltration was observed.

Finally, the feasibility of IL-4 for treatment of Graves' disease was evaluated. Groups of hyperthyroid mice ($n = 6-7$ in each group) were left untreated or injected i.m. with AdRGDCMVIL-4 (5×10^{10} particles/mouse) alone or in combination with AdCMVTSHR (10^{11} particles/mouse) twice at a 2-wk interval. Serum T_4 was measured 2 wk after the second immunization. Although the disease remitted spontaneously in some mice, the incidence of disease and mean T_4 levels were not significantly different after treatment in three groups (Fig. 6).

Discussion

We have investigated the role of IL-12 and IL-4, Th1 and Th2 cytokines, respectively, in the development of Graves' hyperthyroidism in our recently established novel murine model (15). In this model, multiple i.m. injections of TSHR-expressing adenovirus induce stimulating TSHR Abs and hyperthyroidism in >50% of female BALB/c mice. Appearance of anti-TSHR Abs of both IgG1 and IgG2a subclasses measured by ELISA and preferential secretion of IFN- γ , not IL-4, from splenocytes in response to TSHR Ag in TSHR-immunized mice reflect the induction of both Th2 and, particularly, Th1 immune responses in this model.

Coinjection of adenovirus expressing IL-4 skewed the development of the immune response against the TSHR toward the Th2 phenotype, as reflected in the decreased ratio of IgG2a:IgG1 TSHR Abs (Th1:Th2) as well as Ag-specific secretion by splenocytes of a Th1 cytokine IFN- γ . This deviation of anti-TSHR immune response away from Th1 domination was accompanied by significant suppression of stimulating TSHR Abs and hyperthyroidism.

In contrast, coinjection of adenovirus expressing IL-12 enhanced IFN- γ secretion from splenocytes, that biasing toward Th1 did not affect induction of stimulating Abs or disease incidence. It is worth noting that Ab titers determined with ELISA and TBIAb assays were not affected quantitatively by expression of either cytokine, suggesting that Ab quality, not quantity, is responsible for disease induction. Similar results have recently been observed in our adenovirus model with different mouse strains (21) as well as for DNA vaccination of outbred mice (9). The significantly lower levels of TSAb and comparable levels of TBAb in the TSHR plus IL-4 group vs the TSHR group indicate that the protective effect of IL-4 is not attributed to the increased proportion of blocking to stimulating Abs. On the other hand, the significantly lower TBAb activities in the TSHR plus IL-12 group than in the other two groups suggest that the Th1/Th2 balance influences the ratio of stimulating/blocking Abs. Overall, these data strongly indicate the crucial role of the Th1 immune response in the pathogenesis of Graves' disease in our model. Incidentally, it is unlikely that the different abilities of IgG1 and IgG2a to bind distinct FcRs and to activate complement (22) contribute to disease pathogenesis.

In this context, it is of interest that the Th2 adjuvant alum, but not the Th1 adjuvant polyribonucleosinic polyribocytidylic acid, completely suppresses Ab production and disease development in another murine Graves' model we have recently established. This model involves injecting female BALB/c mice with bone marrow-derived dendritic cells infected with adenovirus expressing TSHR (23). These data suggesting the importance of Th1 immune responses in Graves' models utilizing TSHR-expressing adenovirus are consistent with our present observations, but are in sharp contrast to the previous studies with the Shimojo model of Graves' disease (10).

These apparently opposing effects of alum in different animal models, along with controversial data regarding the Th1/Th2 paradigm in experimental murine and human disease (reviewed in the Introduction), indicate that it is unwise to conclude that Graves' manifestations are the outcome of Th1-type immune responses. Moreover, the view that Th2 cytokines play a primary role in humoral autoimmune diseases may be unwarranted. Indeed, the importance of Th1 immune responses has been demonstrated in other Ab-mediated organ-specific autoimmune diseases such as autoimmune hemolytic anemia (AIHA), myasthenia gravis, and Goodpasture's disease (24). For example, preferential secretion of Th1 cytokines in response to autoantigens has been demonstrated in AIHA and myasthenia gravis (24, 25). Moreover, amelioration in AIHA was observed when mice were injected with DNA encoding IL-4 or with anti-IL-12 Ab (24). On the other hand, in murine lupus, an Ab-mediated systemic autoimmune disease (26), disease activity was suppressed in IFN- γ and IL-4 KO mice as well as in IL-4-transgenic mice (27, 28). These divergent results are not easy to reconcile and may point to the complexity of cytokine interactions.

Cytokines exert variable actions on the induction/suppression of immune response, depending on the level and location of production or the route and timing of administration. For example, IL-4, which is generally believed to stimulate humoral immunity and inhibit a Th1 immune response (29), is also shown to have the indispensable role in establishment of an early phase of Th1 immune response (30, 31). Divergent effects of certain cytokines have been reported in Th1-dominated, T cell-mediated autoimmune diseases. Thus, the therapeutic efficacy of adenovirus-mediated transiently expressed IL-4 (32) and a pathogenic role of sustained administration of rIL-4 (33) have both been demonstrated in inflammatory bowel disease. Prevention of autoimmune diabetes in nonobese diabetic mice has been achieved with systemic injection

of adenovirus-expressing IL-4 (34) and also in IL-4R KO mice (35).

In conclusion, Graves' disease induced by injecting TSHR-expressing adenovirus is characterized by Th1 and to a lesser extent Th2 responses. Despite its protective effect on induction of murine Graves' disease, IL-4 had no beneficial effect in a therapeutic setting. Skewing an established immune response might be more difficult than inducing a polarized response in naive mice. Indeed, it is reported that IL-4 fails to convert an already established Th1 response into a Th2 response (36). Nevertheless, the pathogenic potential of the Th1-type immune response in Graves' hyperthyroidism suggests the need for caution in developing therapeutic strategies aimed at deviating a presumed Th2-mediated autoimmune response to Th1-dominant immunity. Furthermore, unlike murine Graves' models, Th1-dominated subclinical chronic thyroiditis likely coexists with Graves' disease in humans, as indicated by the presence of autoantibodies against thyroglobulin and thyroid peroxidase in most Graves' sera, which may also worsen with such treatment. Further studies will be required to explore the therapeutic potential of cytokine therapy in humans with Graves' disease.

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