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Title: Expression of Immuno-Regulatory Molecules by Thyrocytes Protects NOD-H2^{h4} Mice from Developing Autoimmune Thyroiditis

Short title: Immuno-regulatory molecules and thyroiditis

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ABSTRACT

One approach to prevent tissue destruction by autoimmune attack in organ-specific autoimmune diseases is to protect the target tissue from autoimmune reaction irrespective of its persistent activity. To provide proof-of-principle for the feasibility of this approach, the immuno-regulatory molecules, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and indoleamine 2, 3-dioxygenase (IDO), were expressed in the thyroid glands using adenovirus vector in non-obese diabetic (NOD)-H2^{h4} mice that spontaneously develop thyroiditis. Mice were anesthetized, and the thyroid glands were exposed by neck dissection followed by *in situ* infection with adenovirus vector (5×10^{10} particles/mouse) twice or thrice, starting one day or four weeks before mice were supplied with NaI water. After 8 weeks of NaI provision, the extent of thyroiditis, serum titers of anti-thyroglobulin (Tg) antibodies and cytokine expression in the spleen were examined. *In situ* infection of adenovirus expressing TRAIL or IDO, but not green fluorescent protein, significantly suppressed thyroiditis scores. However, anti-Tg antibody titers and expression levels of cytokines (IFN- γ and IL-4) in the spleen remained unaltered. Importantly, adenovirus infection 4 weeks after NaI provision was also effective at suppressing thyroiditis. The suppressive effect of TRAIL appears to be mediated at least partly by accumulation of CD4⁺Foxp3⁺ regulatory T cells into the thyroid glands. Thus, localized expression of immuno-regulatory molecules efficiently protected the thyroid glands from autoimmune attack without changing the systemic autoimmunity in NOD-H2^{h4} mice. This kind of immunological intervention, although it does not suppress autoimmune reactivity, may have a potential for treating organ-specific autoimmune diseases.

INTRODUCTION

There appear to be two distinct approaches to prevent destruction of the target tissues by autoimmune attack in organ-specific autoimmune diseases. One is to dampen the abnormally activated autoantigen-specific immune responses, and the other to protect the target tissues from autoimmune attack irrespective of persistent autoimmunity. The feasibility of the latter approach can be investigated by localized expression of the immuno-regulatory molecules in the target tissues of animal models of organ-specific autoimmune diseases. In this case, immuno-suppression would be limited to the microenvironments surrounding the tissue of expression.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (1) and indoleamine 2, 3-dioxygenase (IDO) (2) are the immuno-regulatory molecules recently identified. TRAIL is a member of the TNF super-family and is constitutively expressed in a variety of cell types including lymphocytes and natural killer cells. Although TRAIL was first reported to selectively kill tumor cells or virus-infected cells by inducing apoptosis, recent studies

reveal that TRAIL also suppresses or kills activated T cells (1, 3, 4). Indeed genetic disruption of TRAIL gene or chronic TRAIL blockade by soluble death receptor 5, one of the receptors for TRAIL, exacerbates autoimmune diseases such as collagen-induced arthritis and streptozocin-induced diabetes in mice (5, 6). Conversely, TRAIL-expressing professional antigen-presenting dendritic cells (DCs) suppress immune responses in autoimmune disease and graft-versus-host disease (3, 4, 7).

IDO is a rate-limiting enzyme in the catabolism of tryptophan, and is expressed in a series of tissues, particularly in lymphoid organs and placenta (2). IDO was originally shown to inhibit proliferation of intracellular pathogens and tumor cells through consumption of the essential amino acid tryptophan. Subsequently, however, immunosuppression has been shown to be an additional role for IDO (2). Importantly, maintenance of pregnancy is associated with a role for tryptophan catabolism in preventing the proliferation of allogenic T cells against paternal antigens expressed on fetus (8) and the expression of IDO in antigen-presenting cells suppresses

autoreactive T cells (9). Local tryptophan deficiency and accumulation of its metabolites such as kynurenine by IDO are thought to exert its action (5). 1-methyl-tryptophan, a pharmacological inhibitor of IDO, has been shown to exacerbate experimental autoimmune encephalomyelitis and autoimmune colitis (10, 11), indicating IDO to be a potent immuno-regulator in autoimmunity. In contrast, overexpression of exogenous IDO results in immunosuppression and tolerance in animal models of tumor immunity and transplantation (12 - 14).

Non-obese diabetic (NOD)-H2^{h4} mice spontaneously develop anti-thyroglobulin (Tg) autoantibodies and intrathyroidal lymphocyte infiltration when supplied with iodine in the drinking water, and are widely used as a model of autoimmune thyroiditis, one of the common organ-specific autoimmune diseases (15, 16). Autoimmune thyroiditis in these mice is considered to be a T cell-mediated autoimmune disease that results in the destruction of the thyroid follicles (17).

The present study was thus designed to study the effect of localized expression of the above-mentioned immuno-regulatory molecules, TRAIL and IDO, on development of thyroid autoimmunity in NOD-H2^{h4} mice, by using replication-defective recombinant adenovirus vector as a gene delivery vehicle.

We here show adenovirus-mediated thyroidal expression of TRAIL or IDO provides an efficient immunological shield against thyroiditogenic autoimmune process without altering the systemic immune system. Thus this kind of immunological intervention may have a potential for treating organ-specific autoimmune diseases.

MATERIALS AND METHODS

Mice

NOD-H2^{h4} mice (15, 16) were obtained from Jackson Laboratory Inc. (Bar Harbor, ME, USA) and bred in the animal facility at Nagasaki University in a specific pathogen-free condition. Both male and female mice were used for the current study. Animal care and all experimental procedures

were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Adenovirus vectors

The cDNA for mouse TRAIL was kindly provided by Dr. S. Hirata at Kumamoto University, Japan (3). The cDNA for mouse IDO was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA extracted from splenocytes stimulated with 200 U/ml IFN- γ for 48 h, followed by sequence validation.

Recombinant replication-defective, fiber-mutant adenoviruses expressing TRAIL, IDO or green fluorescent protein (GFP, as a control) (Ad-TRAIL, -IDO or -GFP, respectively) were constructed, amplified and purified as previously described (18). Infectivity of these fiber-mutant adenoviruses to a variety of cells is higher than that of wild type adenovirus (19).

In vitro and in situ infection of adenovirus

For *in vitro* infection, COS cells were infected with Ad-TRAIL, -IDO or -GFP at a multiplicity of infection (MOI) of 10,000 particles/cell for 24 h.

For *in situ* infection, NOD-H2^{h4} mice (6 to 8 weeks old) were anesthetized with pentobarbital. An approximately one cm-long midline incision was made on the anterior neck under sterile conditions. The underlying submandibular salivary glands were separated to the both sides to visualize laryngotrachea and strap muscles. The strap muscles were then cut to form a gutter in which 20 μ l PBS with/without adenovirus (5×10^9 or 10^{10} particles/mouse) was applied. This procedure allowed the direct contact of adenovirus with the thyroid glands. After a few minutes of contact, the submandibular glands were returned to their normal position, and the skin incision was closed using conventional surgical procedures (20).

Detection of TRAIL and GFP expression

In vitro expression of TRAIL and GFP in COS cells of mock-infected or infected with Ad-IDO or -TRAIL was analyzed on a

FACScan flow cytometry using CellQuest software (BD Biosciences PharMingen, San Diego, CA). Phycoerythrin-conjugated anti-TRAIL monoclonal antibody (clone N2B2, eBioscience, San Diego, CA, USA) was used to stain the cells expressing TRAIL.

For detection of *in situ* expression of TRAIL, mice were infected with adenovirus (5×10^{10} particles/mouse), and 3 days later the thyroid cells were prepared by enzymatic digestion from the thyroid tissues as previously described (21) and seeded on 10-cm dishes. On the next days, the isolated thyroid cells were harvested and analyzed on a FACScan flow cytometry as described above.

In situ expression of GFP was examined in the thyroids removed at 3 days, and 1, 4 and 8 weeks after infection by a confocal laser scan microscope LSM5Pascal (Carl Zeiss, Tokyo, Japan).

Detection of IDO expression

IDO expression was determined by western blotting with 20 μ g of total cell lysates prepared from COS cells of mock-infected or infected with Ad-IDO (for *in vitro* expression), or 40 μ g from mock-infected or Ad-IDO infected thyroid tissues (for *in situ* expression). The signal was developed with polyclonal anti-IDO polyclonal antibody (22) and alkaliphosphatase ABC method (Vectastatin ABC kit, Vector Laboratories, Burlingame, CA).

Induction of thyroiditis

Mice were supplied with 0.15 % sodium iodine (NaI) in the drinking water. Groups of mice were infected with adenovirus twice (1 day before and 4 weeks after the beginning of NaI provision in the drinking water) or thrice (4 weeks and 1 day before, and 4 weeks after, NaI supply) (see Fig. 2, A). Eight weeks after NaI provision, mice were euthanized, and the thyroid glands, blood and the spleen were harvested to determine the extent of thyroiditis, the titers of serum anti-Tg autoantibodies and cytokine expression levels, respectively.

In a therapeutic setting, adenovirus was infected to the thyroids on one occasion 4

weeks after exposure of the mice for NaI.

Evaluation of thyroiditis

Thyroid tissues were fixed in 10 % formalin and embedded in paraffin. Five-mm-thick sections were prepared and stained with hematoxylin and eosin (H & E). Thyroiditis was assessed for extent of lymphocyte infiltration as follows (23); grade 0, normal thyroid; grade 1, less than 10 % lymphocytic infiltration of the thyroid; grade 2, 10 to 30 % lymphocytic infiltration; grade 3, 30 to 50 % lymphocytic infiltration; grade 4, greater than 50 % lymphocytic infiltration. The final thyroiditis scores were expressed as means of at least 3 noncontiguous sections from each thyroid gland.

ELISA assay for anti-Tg antibodies

Tg was purified from mouse thyroid glands as previously described (23). ELISA wells were coated overnight with 100 μ l Tg protein (10 μ g/ml) and incubated with mouse sera (1:300 dilution). After incubation with horseradish peroxidase conjugated anti-mouse IgG (BD Biosciences PharMingen), color was developed using orthophenylene diamine and H₂O₂ as substrate and optical density (OD) read at 492 nm.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA (1 μ g) was extracted from the spleen using Isogen (WAKO, Tokyo, Japan) and reverse-transcribed to generate cDNA with SuperScript III (Invitrogen, Carlsbad, CA) and oligo-dT. PCR was then performed with PrimeSTAR HS DNA polymerase (Takara, Tokyo, Japan) and the following primer pairs;

IFN- γ forward primer: 5'-CACGGCACAGTCAATGAAAG-3';

IFN- γ reverse primer: 5'-CCTTGCTGTTGCTGAAGAAG-3';

IL-4 forward primer: 5'-CCTGCTCTTCTTTCTCGAATGT-3';

IL-4 reverse primer: 5'-TTTCAGTGATGTGGACTTGGAC-3';

β -actin forward primer: 5'-CTGAACCCTAAGGCCAACCGTG-3';

β -actin reverse primer: 5'-AGCTCTTTGAGGTGCTCCAG-3'.

The intensity of specific bands for IFN- γ and IL-4 on an agarose gel electrophoresis was quantified by NIH image J software. Expression levels of β -actin for each sample were used for data normalization.

Immunohistochemistry

The thyroid glands were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen and cut by a cryostat into 6- to 7- μ m-thick sections.

CD4 and Foxp3 staining was performed as recently described (24). Briefly, after fixing with cold acetone for 10 min at 4C and blocking non-specific reactions and endogenous biotin activity with Blocking One (Nacalai Tesque, Kyoto, Japan), the samples were incubated with the primary antibodies [rabbit anti-mouse Foxp3 (25) and/or hamster anti-mouse CD4 (H129.19; BD Biosciences Pharmingen)] for 1 h at room temperature and subsequently with the secondary antibodies (Alexa Fluor 555 goat anti-rabbit IgG and/or Alexa Fluor 488 goat anti-hamster IgG, both from BD Biosciences Pharmingen) for 1 h at room temperature, and then fixed for 10 min at 4 C in PBS containing 4% paraformaldehyde.

For TUNEL staining, the sections were incubated with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP at 37 C for 60 min, according to the manufacturer's instruction (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

All sections were analyzed with a confocal laser scan microscope LSM5Pascal (24). Numbers of CD4⁺ T cells and CD4⁺Foxp3⁺ T cells, and of apoptotic cells were counted in 4 nonconsecutive microscopic fields in each tissue.

Statistical analysis

Thyroiditis scores, and levels of antibodies and cytokine expression were analyzed by Student's t-test. A 'p' value of less than 0.05 was considered statistically significant.

RESULTS

In vitro expression of IDO, TRAIL and GFP in COS cells infected with adenovirus

To confirm that adenovirus vectors we generated can express the transgenes, COS cells were infected with adenovirus at a MOI of 10,000 particles/cell. One day later, the cells were harvested and subjected to western blotting or flow cytometric analysis. Approximately 40 kDa IDO protein was clearly detected in the cells infected with Ad-IDO, and not in Ad-GFP infected cells, by western blotting (Fig. 1, A). Expression of TRAIL and GFP was also confirmed by flow cytometry in the cells infected with Ad-TRAIL and Ad-GFP, respectively (Fig. 1, B).

In situ expression of GFP in the thyroid glands

To determine *in vivo* infectivity of adenovirus, the thyroid glands were directly infected with Ad-GFP (5×10^9 or 10^{10} particles/mouse) as described in the *Materials and Methods* and removed 3 days, and 1, 4 and 8 weeks later. GFP expression was readily observed throughout the thyroid glands infected with higher amounts of Ad-GFP (5×10^{10} particles) 3 days (supplementary Fig. S1), and 1 and 4 weeks (not shown), but not 8 weeks, after infection. However, no fluorescence was detected in the thyroid glands infected with lower amounts of Ad-GFP (5×10^9 particles) at any time point. Thus adenovirus-mediated transgene expression with 5×10^{10} particles/mouse is detectable and persists at least for 4 weeks. Therefore, the higher amounts of adenovirus were used in all the subsequent experiments.

In situ expression of IDO and TRAIL was also confirmed. As for *in vitro* expression data, approximately 40 kDa IDO protein was detectable in western blotting with the lysates from the Ad-IDO infected thyroid tissues (Fig. 1, C). Furthermore, TRAIL expression was also demonstrable in flow cytometry with thyroid cells enzymatically isolated from the Ad-TRAIL infected thyroid glands (Fig. 1, D).

Suppression of thyroiditis, but not systemic anti-thyroid autoimmune responses, by in situ infection of Ad-TRAIL or Ad-IDO

In the first series of experiments, the

thyroids were infected *in situ* with Ad-TRAIL or Ad-IDO twice (1 day before and 4 weeks after beginning of NaI provision in the drinking water) (the solid arrows in Fig. 2, A). Infection of Ad-TRAIL or Ad-IDO significantly suppressed thyroiditis. Thus the thyroiditis scores were decreased from 3.73 ± 0.46 (sham-operated mice, mean \pm S.D.) to 2.18 ± 1.22 ($p < 0.0001$) by Ad-TRAIL and to 1.57 ± 1.11 ($p < 0.0001$) by Ad-IDO (Fig. 2, B) ($n = 15$ for each group). Infection of Ad-GFP did not influence thyroiditis scores. The representative histology of thyroid glands was shown in the Fig. 3; normal thyroid gland in a naive mouse (A), grade 2-thyroiditis in an Ad-TRAIL infected mouse (B) and grade 4-thyroiditis in an Ad-GFP infected mouse (C).

In the second set of experiments in which adenovirus infection was performed thrice starting 4 weeks before provision of NaI (the open arrows in Fig. 2, A), the suppressive effect appeared more profound than the first experiment; thyroiditis scores were declined from 3.43 ± 0.59 to 1.74 ± 1.02 ($p < 0.0001$) by Ad-TRAIL and to 1.35 ± 0.98 ($p < 0.0001$) by Ad-IDO (Fig. 2, C) ($n = 10$ for each group).

Whether regional expression of the immuno-regulatory molecules affect systemic immune response was investigated by measuring serum anti-Tg antibody titers and cytokine expression in spleen. Anti-Tg-antibody titers (Fig.4, A and B) were 0.56 ± 0.30 , 0.61 ± 0.36 and 0.52 ± 0.28 OD₄₉₂ in sham-operated mice, and those infected with Ad-TRAIL or -IDO, respectively, in the first experiments shown in Fig. 2, B and 0.52 ± 0.38 , 0.49 ± 0.34 and 0.47 ± 0.32 in these 3 groups in the second experiments in Fig. 2, C.

Expression levels of cytokine mRNAs in spleen were 7.21 ± 1.18 versus 7.43 ± 1.26 arbitrary units for IFN- γ and 7.64 ± 1.68 versus 8.30 ± 1.35 for IL-4 in mock-infected and Ad-TRAIL infected thyroids, respectively (Fig. 5). Similarly comparable expression levels of IFN- γ and IL-4 mRNAs were observed between mock-infected and Ad-IDO infected thyroids (data not shown).

Thus, thyroidal TRAIL or IDO expression

did not affect extrathyroidal immune responses (anti-Tg antibody titers and splenic cytokine expression) in both experiments.

Accumulation of regulatory T cells (Treg) in the thyroid glands induced by expression of TRAIL

The mechanisms for TRAIL- and/or IDO-mediated immune suppression include an increase in number of naturally-occurring regulatory CD4⁺CD25⁺ T cells (Treg), a T cell subpopulation that negatively suppresses immune response (4, 26), and induction of apoptosis (5, 6, 14). We therefore compared the ratios of Treg to CD4⁺ T cells and the percentage of apoptotic cells between the thyroids from control and TRAIL- or IDO-treated mice.

T cells double positive for CD4 and Foxp3 were defined as Treg, because Foxp3 is at present the most reliable molecular marker for Treg (27). The ratio of Treg to CD4⁺ T cells remained unchanged in the periphery (data not shown). In contrast, the ratio of Treg to CD4⁺ T cells measured by immunohistochemistry was significantly higher in Ad-TRAIL infected thyroids than in mock-infected thyroids: 16.4 ± 3.8 versus 4.8 ± 2.8 % (mean \pm S.D., $n=3$) ($p < 0.01$). These data indicate TRAIL-induction of Treg accumulation into the thyroid glands. Representative photographs for immunofluorescence are shown in Fig. 6. In contrast, IDO had no effect on Treg accumulation in the thyroids (data not shown).

Turning to induction of apoptosis, the number of apoptotic cells (lymphocytes and thyrocytes) was determined by TUNEL assay. No difference was observed between mock-infected and Ad-TRAIL or Ad-IDO infected thyroids (data not shown).

Therapeutic usefulness of in situ infection of Ad-TRAIL

To evaluate the therapeutic potential of *in situ* infection by adenovirus expressing the immuno-regulatory molecules, mice were infected once with Ad-TRAIL 4 weeks after the beginning of NaI. Anti-Tg antibodies were readily detected at this time point (data not shown), indicating that the anti-thyroid immune response is already established. As

shown in Fig. 7, Ad-TRAIL moderately but significantly decreased the degree of thyroiditis (3.29 ± 0.80 to 2.56 ± 0.73 , $n = 15$ for each group; $p < 0.05$). Thus *in situ* expression of TRAIL can also be therapeutically useful.

DISCUSSION

In the present study, we hypothesized that localized expression of the immuno-regulatory molecules such as TRAIL or IDO, as a therapeutic modality for organ-specific autoimmune diseases, would lead to immuno-suppression limited to the microenvironments surrounding the tissue of expression. To verify our hypothesis, these immuno-regulatory molecules were expressed by recombinant adenovirus in the thyroid glands of NOD-H2^{h4} mice, a strain that provides a spontaneous animal model of autoimmune thyroiditis.

We first showed high efficiency of adenovirus infection to the thyroid glands, which persisted at least for a month. This high infectivity and long-lasting transgene expression may be at least partly attributed to use of the fiber mutant adenovirus as mentioned in the *Materials and Methods* (19, 28). These findings enabled us to examine the effect of *in situ* expression of the immuno-regulatory molecules on thyroiditis in NOD-H2^{h4} mice. We found that infection of Ad-TRAIL or Ad-IDO to the thyroid glands 1 day or 4 weeks prior to providing the mice with iodized water significantly suppressed thyroiditis. These data clearly indicate that *in situ* expression of immuno-regulatory molecules efficiently protects the thyroid glands from autoimmune attack. However, serum anti-Tg antibody titers and expression levels of cytokines IFN γ and IL-4 in the spleen were unaffected. Thus, the effect of intrathyroidal expression of immuno-regulatory molecules is confined to the thyroid glands, and does not impact systemic immunity. It is essential for any treatment modality to avoid unnecessary systemic immune suppression. Furthermore, we found that suppression of thyroiditis was observed when adenovirus was given after anti-thyroid immune response was induced,

indicating that this approach is also therapeutically effective. This finding is a highly critical issue in a clinical setting. Consequently, this type of immunological intervention may have a potential for treating organ-specific autoimmune diseases.

Similar studies have previously been conducted in an inducible mouse model of autoimmune thyroiditis by using IL-10 and Fas ligand as immuno-suppressive molecules and DNA plasmid as a gene delivery vehicle (29, 30). Surprisingly, despite much lower efficacy of gene transfer by non-viral plasmid vector than by adenovirus, local expression of IL-10 or Fas ligand by plasmid suppressed not only intrathyroidal lymphocyte infiltration but also systemic anti-thyroid immune response. These data are contradictory to our current findings. It is unclear how these distinct results can be reconciled at present.

Adenovirus-mediated localized IDO expression has recently been shown to be effective at (i) protecting the transplanted islets from NOD diabetogenic T cells (12) and (ii) suppressing allograft rejection in lung transplantation (13). In addition, intra-articular adenoviral-mediated TRAIL expression has also been shown to be beneficial for suppressing arthritis (6, 31). As for our present report, these studies were also conducted as attempts to control abnormal autoimmune responses locally.

The mechanisms of actions of TRAIL and IDO on immune system are wide-ranging. One of the common mechanisms is engagement of Treg (4, 26). For examples, Hirata *et al.* (4) reported that TRAIL⁺ DCs activate and augment proliferation of CD4⁺CD25⁺ T cells, and Fallarino *et al.* (26) showed that naive T cells differentiate to Treg by co-culture with IDO⁺ DCs. Our results are consistent with those of Hirata *et al.* (4) in that TRAIL increased the ratios of Treg to CD4⁺ T cells in the lesion. Wang *et al.* (32) studied the effect of systemic administration of TRAIL protein on thyroiditis in a transfer model of thyroiditis in mice. As expected, injection of TRAIL suppressed not only thyroiditis, but also splenocyte proliferation and IFN- γ production, as well as serum anti-Tg antibody titers. In their more recent study, TRAIL is also reported to enhance

CD4⁺CD25⁺CD45RB^{low} Treg (33). However, in our study, accumulation of Treg was not observed when IDO was expressed in non-professional antigen presenting thyroid cells.

Some, but not all (5, 6, 14), publications reported that TRAIL and IDO also induce apoptosis and/or inhibit proliferation in lymphocytes. In arthritis models, for examples, one group (31) demonstrated apoptosis induction by TRAIL in abnormally proliferating synovial cells in a rabbit model, while another group (6) showed prevention of proliferation of both synovial cells and arthritogenic lymphocytes by TRAIL in a mouse model. In the present study, no significant increase in apoptotic lymphocytes/thyocytes by TRAIL or IDO was observed. Therefore, all the data suggest that accumulation of Treg may be a mechanism for suppression of thyroiditis by TRAIL, and that IDO may exert its inhibitory action by suppressing proliferation of lymphocytes rather than inducing apoptosis or accumulating Treg in our study.

One potential disadvantage of our study is transient expression of the transgene induced using adenovirus. It should be however emphasized that our investigation was performed as a proof-of-principle experiment and did not examine very long-term effect of adenovirus-mediated

expression of immuno-regulatory molecules on thyroiditis. Permanent transgene expression by other viral vectors such as retrovirus or adeno-associated virus may be more feasible in the future. Another drawback is the relatively small therapeutic effect we observed as shown in Fig. 7. However, disease progress may be much faster in NOD-H2^{h4} mice as compared to the natural disease development of human Hashimoto's thyroiditis. Consequently, the efficacy of our approach may be more pronounced when tested in a clinical setting.

Finally, we conclude that this type of immunological intervention, although it does not suppress the autoimmune reaction itself, has the potential for treating organ-specific autoimmune diseases. Further studies will clearly be necessary prior to clinical application.

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FIGURE LEGENDS

Figure 1. *In vitro* and *in situ* expression of TRAIL, IDO and GFP proteins. In *A* and *C*, the cell lysates from COS cells infected with Ad-IDO or Ad-GFP (negative control) (*A*) or the thyroid glands of mock-infected or infected with Ad-IDO (*C*) were subjected to western blotting as described in the *Materials and Methods*. In *B* and *D*, COS cells of mock-infected or infected with Ad-TRAIL or Ad-GFP (*B*) or thyroid cells isolated from the thyroids of mock-infected or infected with Ad-TRAIL (*D*) were analyzed by flow cytometry as described in the *Materials and Methods*.

Figure 2. Suppression of thyroiditis in NOD-H2^{h4} mice infected with Ad-TRAIL or Ad-IDO. *A*, Schedule for investigating the effect of adenovirus infection on thyroiditis. Solid and open arrows depict the timing of adenovirus infection in *B* and *C*, respectively. Mice were mock-infected or infected with Ad-GFP, Ad-TRAIL or Ad-IDO twice (*B*) or thrice (*C*) and were maintained in the presence or absence of NaI in the drinking water for 8 weeks. *B* and *C*, The thyroid glands were examined with H & E staining, and the thyroiditis scores were assessed as described in the *Materials and Methods*. *, $p < 0.01$.

Figure 3. Representative histology of the thyroid glands in NOD-H2^{h4} mice. *A*, grade 0 in a naïve mouse, *B*, grade 2-thyroiditis in Ad-TRAIL treated mouse, *C*, grade 4-thyroiditis in Ad-GFP treated mouse.

Figure 4. Lack of effect of Ad-TRAIL or Ad-IDO infection on anti-Tg antibody titers. Anti-Tg antibody titers in sera from the mice shown in the Fig. 2, *B* and *C* were determined by ELISA (see the *Materials and Methods*) and are shown in *A* and *B*, respectively.

Figure 5. Lack of effect of Ad-TRAIL or Ad-IDO infection on expression levels of mRNAs for IFN- γ (*A*) and IL-4 (*B*) in the thyroid glands. Total RNA extracted from the thyroid glands from mice of mock-infected or infected with Ad-TRAIL were subjected to RT-PCR as described in the *Materials and Methods*. *A* and *B*, representative pictures for IFN- γ , IL-4 and β -actin mRNA expression in control and Ad-TRAIL infected thyroids. *C* and *D*, quantification of expression levels of IFN- γ and IL-4 mRNAs normalized by β -actin mRNA expression. Means \pm S.D. ($n=3$).

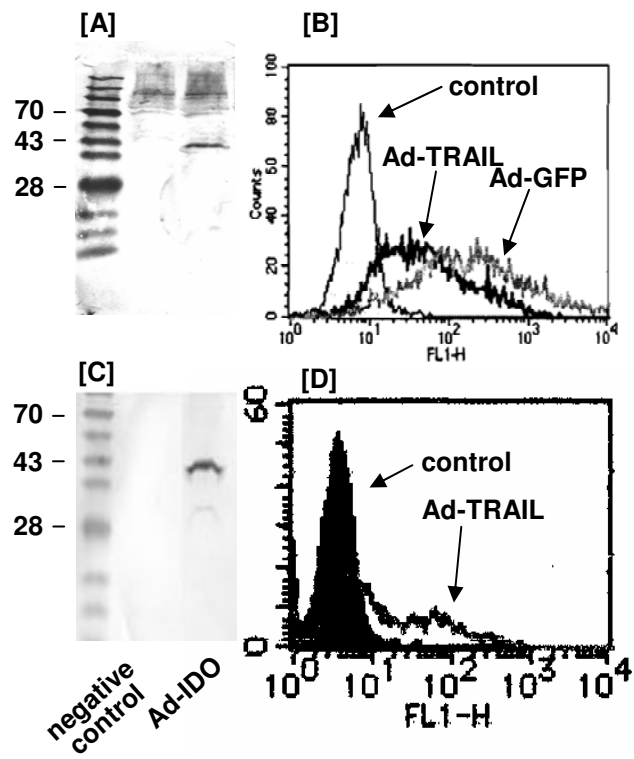
Figure 6. Expression of CD4 and Foxp3 in the thyroid glands. The mock-infected (*A*) or Ad-TRAIL-infected (*B*) thyroid glands were stained with anti-CD4 and anti-FoxP3 antibodies as described in the *Materials and Methods*. Green and red indicate CD4 and Foxp3, respectively, expression. Magnification, x400.

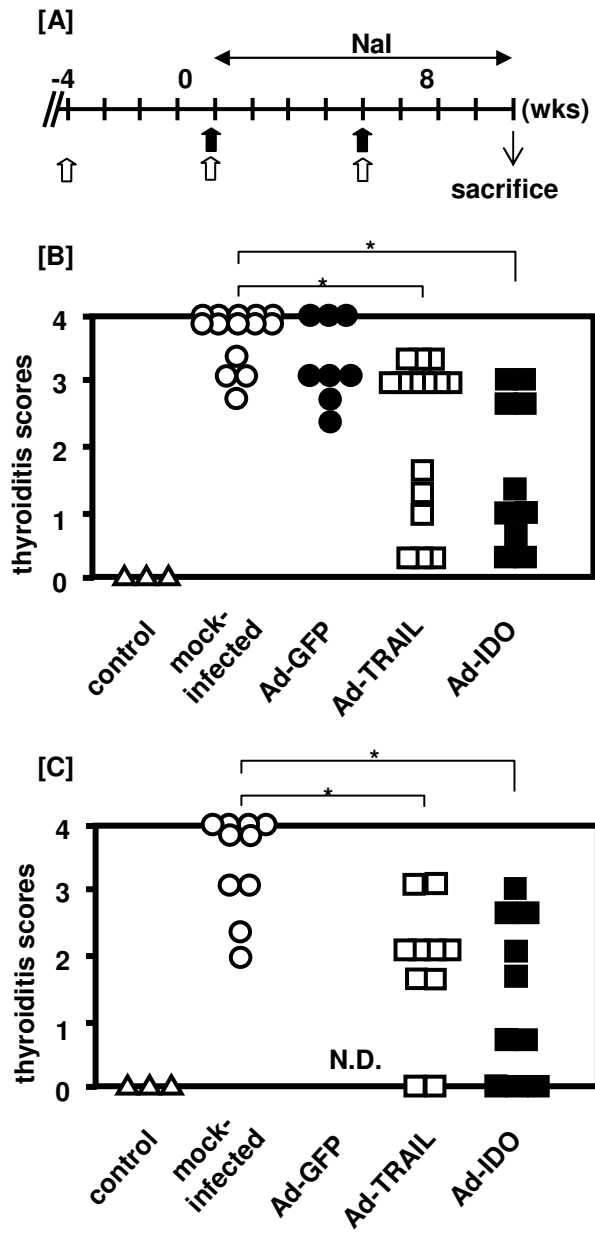
Figure 7. Therapeutic usefulness of Ad-TRAIL. Mice were mock-infected or infected with Ad-TRAIL 4 weeks after exposure to NaI in the drinking water and maintained on iodized water for an additional 4 weeks. The thyroid glands were then examined with H & E staining, and the thyroiditis scores were assessed as described (the *Materials and Methods*). The individual values and means \pm S.D. are shown. *, $p < 0.05$.

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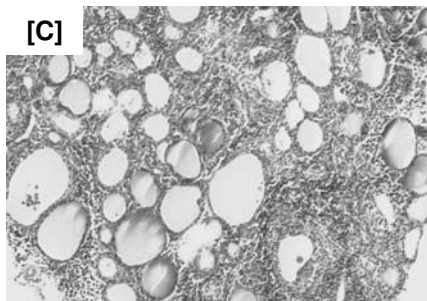
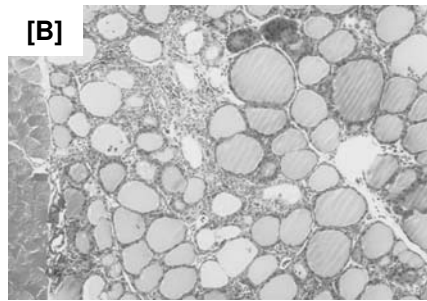
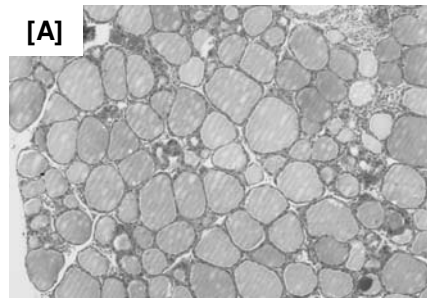
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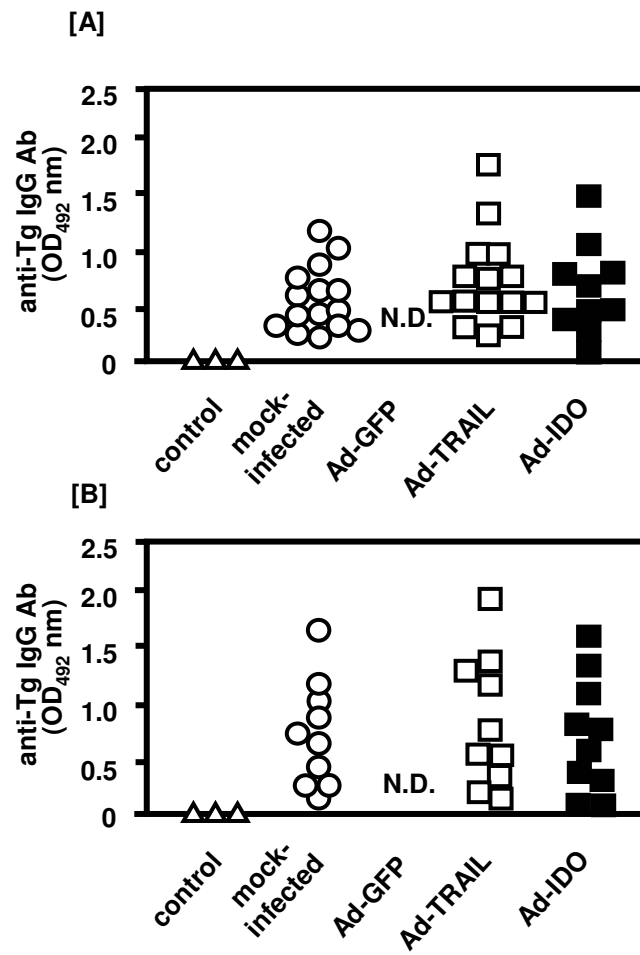
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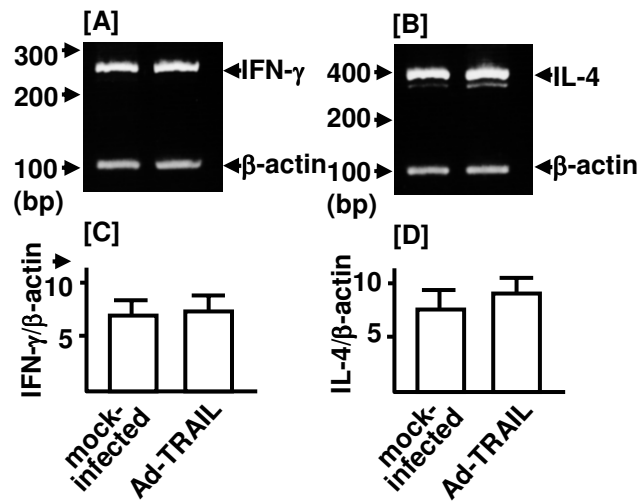




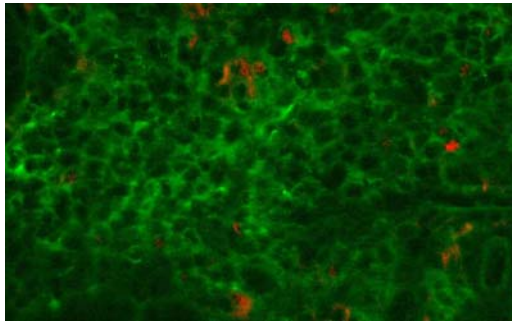
Nakahara et al. Figure 2







[A] control



[B] Ad-TRAIL-treated

